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PERFORMANCE OF THE BLACK TIGER PRAWN (*PENAEUS MONODON*) FED FUCOIDAN UNDER SUB-OPTIMAL CONDITIONS

by

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ABSTRACT

The Australian wild caught prawn sector has been in a state of steady decline in terms of tonnages and value over the past decade; while the aquaculture sector for prawns has been increasing over the same period. Prawn production within the aquaculture sector accounted for 6% of the total aquaculture production value for 2012-13. The industry does experience periods of extreme environmental conditions due to the geographic location where prawns are farmed, causing financial loses. These extreme conditions include high temperatures and heavy rainfall due to onset of the monsoonal season. Environmental stress caused by acute changes in temperature and salinity impact upon the immune system and aerobic metabolism of prawns, increasing susceptibility to disease, such as Gill-Associated Virus (GAV). Immunostimulants such as fucoidan and β-glucan have shown potential to increase immune response and survival to diseases in penaeid prawns.

Preliminary screening of commercially available fucoidan products were included in formulated feeds at 0.2 $g.kg^{-1}$ (*Undaria pinnatifida* [UP85 and F50], *Fucus vesiculosus* [FV90]) and 33 $g.kg^{-1}$ (*Ascophyllum nodosum*, MMB), and were fed to juvenile *Penaeus monodon* twice daily for 15 d to determine effects on growth performance, immune response and digestive gland (DG) histology. At the conclusion of the experiment the mean feed intake (FI), specific growth rate (SGR), weight gain (WG), feed efficiency ratio (FER) and survival were not different between feed treatments. Total haemocyte count (THC) and phenoloxidase activity (PO activity) did not increased in *P. monodon* fed fucoidan, although prawns fed the FV90 had increased granular haemocyte count (GHC) compared to prawns fed F50. DGs presented as tightly packed tubules contained within the inter-connective tissue with star-shaped tubule lumens, indicative of healthy animals.

In another study *P. monodon* were fed treatments four times daily containing FV90, UP85 and F50 at an inclusion level of 1g.kg^{-1} of fucoidan for 21 d. *P. monodon* were cultured at 30°C water temperature and 40‰ salinity, until the conclusion of the growth trial were prawns were subjected to either an environmental stressor (24°C and 20‰) or held under non-stress conditions (30°C and 40‰) (control) for 3 hours. Haemolymph was removed for determining immune response. At the conclusion FI, SGR, WG, survival and whole body chemical composition (crude protein, crude lipid and gross energy) of *P. monodon* were not different between feed treatments. A combined drop in water temperature and salinity elicited a decrease in THC and GHC when compared to non-stress prawns; however there was no effect of environmental stress on the PO activity.

The effect of feeding fucoidan (1 g.kg^{-1}, FV90) on the aerobic metabolism ($\dot{M}o_2$) and immune response (THC, GHC, PO activity) of juvenile and sub-adult *P. monodon* was determined. Juvenile prawns were subjected to an acute decrease in water temperature (30-24°C) and salinity (40-20‰) over a 6 h period, before measuring $\dot{M}o_2$ (standard metabolic rate [SMR], routine metabolic rate [RMR], maximal metabolic rate [MMR], active metabolic rate [AMR] and aerobic scope [A-SCOPE]). Juvenile prawns had a significantly higher AMR compared to prawns fed the control feed. Sub-adult prawns were subjected to the same decrease in water temperature and salinity; however a control group was included where water parameters remained at 30°C and 40‰. After 6 hours, sub-adult prawns were further acclimated to their final water parameters for 72 hours to simulate a chronic stress, prior to measuring $\dot{M}o_2$. There were no interactions between feed treatment and stressors for $\dot{M}o_2$. When pooled by stressor, non-stress prawns had significantly higher $\dot{M}o_2$ values, when compared to prawns held under stress conditions. There were no significant interactions of feed and stress treatments on the

THC, GHC and PO activity of prawns. Data pooled within stress and control groups for immune response analyses (THC, GHC and PO activity), determined prawns held under stress conditions had significantly higher values. On average stressed prawns had 25% more THC and 39% more GHC than prawns held under optimal conditions and PO activity of stressed prawns was on average 61.7% higher than prawns held under optimal conditions.

To determine the effect of fucoidan (FV90) and SANICTUM® (β-glucan and peptidoglycan) in feeds for *P. monodon* on the growth performance, immune response and survival after challenge with White Spot Syndrome Virus (WSSV), three experiments were conducted. After 28 d of feeding there were no differences in WG, SGR and survival. The THC, GHC and PO activity of prawns were also not different between feed treatments. Prawns inoculated with WSSV via intramuscular injection (IM) and reverse gavage (RG) were sampled for THC, GHC and PO activity 24 h post-infection. There was no interaction of diet and inoculum type on THC for IM. Pooled THC data showed prawns inoculated with WSSV had significantly less THC and GHC compared to PBS controls. Prawns fed the basal and FV90 feed treatments had significantly lower PO activity than the PBS control group. Prawns inoculated with WSSV via RG showed that basal, FV90 and UP85 fed prawns had significantly less THC than prawns inoculated with PBS control. Prawns inoculated with WSSV via RG had significantly less GHC, although this did not manifest in a decrease in PO activity.

For the challenge trial, prawns fed the basal feed and infected with WSSV via IM suffered 100% mortality by day 7. However, prawns fed immunostimulants had 11.11% survival by day 7. When prawns were inoculated with WSSV by RG the overall survival was high (62-70%) by day 21. WSSV concentration of prawns inoculated by IM was not different between prawns fed all

feed treatments. However, in the RG group the viral concentration was below the detection sensitivity of the PCR assay.

This research showed that feeding *P. monodon* feeds containing fucoidan had no negative impacts on growth rates, immune response or DG health. Using the current concentrations of fucoidan and SANICTUM® in *P. monodon* feeds under the protocols used there were no effects of the immunostimulants on growth or immune response compared to prawns fed the basal control or commercial feed treatments. There were also no negative effects on feed intake or survival of *P. monodon*. There was a consistent decrease in THC and GHC with environmental stress. Juvenile prawns fed fucoidan had an increased AMR when subjected to environmental stress, which was not determined in sub-adult prawns. The RG method protocol did not elicit a detectable infection level by PCR in the survival challenge trial and further improvement is required for conducting disease challenges using a more natural infection route and for assessing immunostimulant products which show promise of viral adsorption with the digestive tract.

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ABBREVIATIONS

∆ Abs/min	Change in absorbance per minute
AAS	Atomic absorption spectroscopy
AMR	Active metabolic rate
ANOVA	Analysis of variance
A-SCOPE	Aerobic scope
AWG	Average weekly gain
bw.d	Body weight per day
cm	Centimetres
d	Day
DG	Digestive gland
df	Degrees of freedom
DHC	Differential haemocyte count
DM	Dry matter
DO	Dissolved oxygen
DRT	Digital recycling timer
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F50	*Undaria pinnatifida*, 50% fucoidan
FCR	Food conversion ratio
FER	Feed efficiency ratio
FI	Feed intake
FV90	*Fucus vesiculosus*, 90% fucoidan
G	Gauge
g	Gravity
g	Gram
g CP.MJ GE	Grams crude protein per mega joule gross energy
GAV	Gill-associated virus
g.kg	Grams per kilogram
GE	Gross energy
GHC	Granular haemocyte count
h	Hour
ha	Hectare
IM	Intramuscular injection
INC	Incorporation
kW	Kilowatt
L	Litres
Ld	Lipid deposits
L-DOPA	L-dihydroxyphenylalanine
L/min	Litres per minute
LGH	Large granular haemocytes
Lu	Lumen
M	Molar
m^{-2} s	Square metres per second
mg	Milligram

mg.kg	Milligrams per kilogram
mg/L	Milligrams per litre
min	Minute
MJ.kg	Mega joules per kilogram
mm	Millimetres
mM	Millimolar
mm^2	Millimetre squared
MMB	Macroalgae blend meal
MMR	Maximal metabolic rate
$\dot{M}o_2$	Aerobic metabolism
mOsm kg	Milliosmoles per kilogram
N	Number of samples
NAPS	Nucleic acid preservation solution
O_2	Oxygen
Optodes	luminescent dissolved oxygen probes
PAMPs	Pathogen-associated molecular patterns
PAR	Photosythetically active radiation
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pd	Prawn day
PL	Postlarvae
PO	Phenoloxidase
ppm	Parts per million
proPO	Prophenoloxidase
PRRs	Pattern recognition receptors
PRPs	Pattern recognition proteins
Pty Ltd	Propriety Limited
PVC	Poly vinyl chloride
RG	Reverse gavage
RMR	Routine metabolic rate
RNA	Ribonucleic acid
S.E	Standard error
S.D	Standard deviation
SGR	Specific growth rate
SGH	Small granular haemocytes
SMR	Standard metabolic rate
SPSS	Standard package for the Social Sciences
SSS	Shrimp salt solution
THC	Total haemocytes count
UP85	*Undaria pinnatifida*, 85% fucoidan
UV	Ultraviolet
W	Watt
WG	Weight gain
WSSV	White spot syndrome virus
WW	Wet weight
x	Times

µl	Microlitre
µm	Micrometre
µmol	Micro mole
‰	Parts per thousand

General Introduction

CHAPTER I

1.1 Prawn aquaculture in Australia

Australian farmed penaeid prawn industry was valued at $60 million (4000 tonnes) for the period 2012-13 and accounted for 6% of the total aquaculture production in Australia (ABARES 2013). The majority of prawn farming is conducted in Queensland, ranging from the Gold Coast up to Mossman in northern Queensland, while a small amount of production also occurs in northern New South Wales. The main species farmed in Australia is the black tiger prawn (*Penaeus monodon*), with growing production also of the banana prawn (*Fenneropenaeus merguiensis*) and limited production of kuruma prawn (*Marsupenaeus japonicus*). Production of prawns in Australia commonly occurs in earthen ponds of 1 ha in size and depths of 1.5 m. To date *P. monodon* hatcheries are mainly reliant on wild broodstock for producing postlarvae in Australia (Mohr et al., 2015). However, advances in domestication and selective breeding of *P. monodon* have occurred within the last few years (Preston et al., 2010).

1.2 Viral diseases of cultured prawns

Intensive prawn culture involves holding animals at high stocking densities within a defined body of water which increases the risk of disease prevalence and the virulence of a pathogen (Chantanachookin et al., 1993). Worldwide, approximately 20 viral diseases have been documented in commercial prawn culture (van de Braak, 2002). Of these viruses; Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Yellow Head Virus (YHV), Taura Syndrome Virus (TSV) and White Spot Syndrome Virus (WSSV), have demonstrated to cause significant mortalities of farmed prawns (Lightner et al., 1983; Boonyaratpalin et al., 1993; Hasson et al., 1995; Lightner, 1996). However, the most significant viral disease to affect prawn production to date is WSSV. First reported in 1992, WSSV has caused economic losses of more

than $7US billion (Lightner, 2003). To overcome stock losses to this disease the use of specific pathogen free (SPF) stocks of *Litopenaeus vannamei* were introduced to Asia, as they were more disease tolerant than *P. monodon* (Flegel et al., 2008). Since the global viral pandemic from 1992 to 2001, production figures have increased significantly, following the introduction of *L.vannamei* into Asia (Lightner, 2003). Australia is free of all the previously listed viral diseases which are significant to prawn farming, except for IHHNV which was isolated in wild prawn stock in 1992 (Owens et al., 1992). However, Australian prawn farmers experience mortality events from Gill-Associated Virus (GAV).

1.2.1 Gill-Associated virus (GAV)

In Australia, the most important viral disease is Gill-Associated Virus (GAV) an RNA nidovirus endemic to New South Wales, Queensland and Northern Territory, which causes sporadic mortality in prawn farming (Spann et al., 1997; Munro et al., 2011). Survival rates of *P. monodon* have been reported at 70% and even lower during the latter periods of production attributed to GAV disease, costing the industry approximately $10 million a year due to mortality and poor market quality (Sellars et al., 2013). GAV presents similarly to YHV, however with no yellowing of the carapace and affects the lymphoid organ tubule structure, digestive gland and gills, where mortality of experimentally infected animals can reach 100% within 3 days (Spann et al., 2003). YHV is differentiated as genotype YHV1 and is closely related to GAV (which is synonymous with YHV2 genotype) and more recently a new genotype YHV7 has been discovered in diseased broodstock from a Queensland hatchery (Cowley et al., 1999; Mohr et al., 2015). GAV is transmitted vertically from chronically infected broodstock;

however horizontal infections are thought to occur through cannibalism and water-borne transmission (Cowley et al., 2002). Generally, prawns in Queensland are presumed to be infected with GAV, although prawns display no clinical signs of disease, there is a negative impact on production rates (Munro et al., 2011). During high monsoonal rainfall events, decreases in both water temperature from 30 to 22°C and salinity from 40 to 10‰ over 2 to 3 days (pers. comm., Matt West, 2011), can initiate an acute viral infection causing significant loss of prawns (de la Vega et al., 2004; Liu et al., 2006; Elliot and Owens, 2015; Mohr et al., 2015; Sellars et al., 2015).

1.3 Prawn immune system

Invertebrates have an innate immune system and cannot increase their immunity by repeated exposures to pathogens or foreign material, as opposed to vertebrates which possess both an innate and an adaptive immune system. However, prawns do have a nucleic acid based adaptive immune system they do not producing antibodies, and therefore do not benefit from the protection provided by vaccination. Invertebrates instead use an array of cellular and humoral immune defence factors to eliminate pathogens and "non-self" particles (Lee and Soderhall, 2002; Cerenius, et al., 2010). The ability of the innate immune system to recognise "self" from "non-self" is achieved by specialised receptors such as molecular pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) which are referred to as pattern recognition proteins (PRPs) (Soderhall et al., 1994; Medzhitov and Janeway, 1997). Although the PRPs recognise the carbohydrate components of the cell walls of invading organisms such as; peptidoglycan (Purivirojkul et al., 2006) in Gram positive and negative bacteria, lipopolysaccharides (Soderhall et al., 1994) from Gram negative bacteria and β-1,3-glucans,

from yeast and double-stranded RNA (viruses), although the proteins alone cannot destroy pathogens (Soderhall et al., 1994; Vargas-Albores and Yepiz-Plascencia, 2000; Lee and Soderhall, 2002).

1.3.1 Cellular response

Prawn cellular defence occurs within the haemocytes (blood cells) and is responsible for encapsulation, nodule formation, phagocytosis, wound repair, clotting and prophenoloxidase (proPO) activity activation (Bachere et al., 1995). There are three types of haemocytes: hyaline, semi-granular and large-granular (Bauchau, 1981). Haemocytes are responsible for sealing off wounds (hyaline cells), phagocytosis (hyaline cells), encapsulation, coagulation, nodule formation and melanisation (small and large granular cells) of pathogens and foreign particles (Smith and Soderhall, 1983; Johansson et al., 2000; Smith et al., 2003).

The total number of haemocytes with the haemolymph is often measured as an indicator of prawn health, because haemocyte numbers change according to stress conditions (Perazzolo et al., 2002), viral disease status (Immanuel et al., 2012), hypoxia (Le Moullac et al., 1998) and immunostimulation (Chang et al., 2003). Furthermore, it has been determined that by measuring the granular haemocyte counts, a more specific measure of defence can be determined because granular haemocytes are responsible for the synthesis and storage of the proPO system (Sritunyalucksana et al., 2005). Small and large granular haemocytes go through a process of degranulation, releasing clotting proteins that activate the proPO system via prophenoloxidase activating enzyme (ppA) to the active form phenoloxidase activity (PO) activity. This chemical reaction uses the ppA enzyme to catalyse the oxidation of phenols to quinones, producing

melanin (Soderhall and Cerenius, 1998). This enzymatic cascade produces melanin, peroxinectin (cell adhesion and peroxidase activity) and production of antimicrobial substances to sequester and eliminate pathogens (Johansson and Soderhall, 1985; Song and Hsieh, 1994; Destoumieux et al., 1997; Sritunyalucksana and Soderhall., 2000). During phagocytosis, haemocytes engulf micro-organisms and produce various reactive oxygen intermediates, by respiratory burst, including superoxide anion, superoxide dismutase, hydroxyl radicals, singlet oxygen, hydrogen peroxide and nitric oxide synthase activity (Roch, 1999; Campa-Cordova et al., 2002). These processes occur quickly upon contact with pathogens and superoxide anion is the first product released from a respiratory burst, and regarded as an accurate measure for quantifying the respiratory burst (Song and Hsieh, 1994; Sritunyalucksana et al., 1999). However, PO activity is thought to be one of the most important biochemical processes occurring within the immune system of prawns, and is widely measured as an immune indictor in studies assessing immunostimulant products prior to disease challenges and after environmental stress, and will be measured within this study as an indicator of immune response.

1.4 Environmental stress

Penaeus monodon are an ectothermic and euryhaline species and can be cultured in a wide range of temperatures and salinities. Pond-reared prawns are exposed to changing environmental conditions which include variations in water temperature, salinity, oxygen and nutrient concentrations which can cause stress. Of these environmental factors, water temperature and salinity are thought be some of the most important abiotic factors (Chen et al., 1995). Environmental stress has demonstrated to reduce immune vigour, such as decreases in

haemocyte counts, PO activity, phagocytic indices and release of free oxygen radicals (Le Moullac and Haffner, 2000; Perazzolo et al., 2002). It also impacts upon the moult cycle, where prawns held above or below optimal temperature and salinity parameters experience extended periods of intermoult or trigger moulting in pre-moult prawns, which reduces growth rates and survival (Staples and Heales, 1991). Finally, environmental stress negatively affects metabolic performance (Chen and Nan, 1993), reduces osmotic and ionic regulation (Mantel and Farmer, 1983), and increases susceptibility of animals to disease (Joseph and Philip, 2007).

1.5 Aerobic metabolism

Metabolic rates in prawns are directly influenced by water temperature and increase exponentially with increasing temperature, within a species-specific physiological thermal range (Brown et al., 2004; Healy and Schulte, 2012). The metabolic rate is determined indirectly in an aquatic medium due to difficulties sampling metabolites and heat loss directly; therefore by measuring the oxygen consumption rate ($\dot{M}o_2$) the aerobic metabolism can be calculated (Brown et al., 2004; Healy and Schulte, 2012), and can be used as an indirect measure of spontaneous energy consumption and can be measured at differing states of activity. The standard and metabolic rate (SMR) is the minimum $\dot{M}o_2$ rate required for life; where the prawn is unfed and an inactive state and has not experienced previous anaerobic exercise, calculated as the lowest 10% of O_2 consumption recordings over a 16 h timeframe, excluding specific dynamic action (Fry and Hart, 1948; Herrmann and Enders, 2000; Clark et al., 2013). Routine metabolic rate (RMR) can be calculated as the overall mean of O_2 consumption recordings, while maximal metabolic rate (MMR) can be defined as an average of the 5 highest O_2 consumption recordings over the same

timeframe (Fitzgibbon et al., 2014). Active metabolic rate (AMR) is the greatest O_2 consumption rate recorded, associated with swimming at a sustained velocity, usually stimulated manually by chasing to exhaustion (Fitzgibbon et al., 2014). The difference in magnitude between SMR and AMR is aerobic scope (A-SCOPE). A-SCOPE provides a measure of the animal's capacity to respond to increased metabolic demands, where a higher A-SCOPE generally indicates increased fitness or ability to respond to changing energetic demands (Bray et al., 1994; Villarreal et al., 2003).

The aerobic metabolism (SMR and AMR) and therefore A-SCOPE of prawns decrease following an acute environmental stress, where changes occur quickly from hours to days (Priede, 1985). Therefore, by measuring the $\dot{M}o_2$, it is possible to determine how well prawns perform under changing environmental conditions, while an increase in $\dot{M}o_2$ provides a higher capacity for prawns to tolerate a stressful event (Chen and Nan, 1993).

1.6 Immunostimulants

Immunostimulants are chemical compounds that stimulate or activate the immune system to increase resistance to pathogens (Raa, 2001). Immunostimulants are commonly derived from; cell wall components of yeasts, fungi and bacteria (β-1,3-glucans and lipopolysaccharides); peptidoglycan; and sulfated fucoidan (algal polysaccharides) from brown and some green seaweed (Liao et al., 1996; Genc et al., 2007; Lahaye and Robic, 2007; Immanuel et al., 2010; Traifalgar et al., 2010). Immunostimulants have functional properties, such as the carbohydrate components of the cell wall, are detected by host pattern recognition proteins (PRPs) and initiate an immune response (stimulation).

The use of oral immunostimulants in prawn feeds is increasing worldwide as they are readily available, and are an alternative to therapeutics, such as antibiotics for increasing survival during disease outbreaks (Raa, 1996; Song et al., 1997; Smith et al., 2003). Immunostimulants have been demonstrated to enhance the immune response in penaeid prawns using both *in-vitro* and *in-vivo* assay methods (Table 1.1).

The timing and method used for administering immunostimulants to aquatic animals, including prawns has received great attention. Feeding immunostimulants after clinical signs of disease are discovered may be too late to save animals, although this may be typical when applying antibiotics (Raa, 2001). In general, it is better to administer immunostimulants before a disease outbreak due to the time required initiating an immune response and reaching peak immunity. Maximum immune response post-feeding varies and has been recorded from 24 d and 30 d respectively for *P. monodon* fed β-1,3-glucan and peptidoglycan (Chang et al., 2000; Purivirojkul et al., 2006), while increased immunity was achieved in *P. monodon* within 15 d fed crude fucoidan (Chotigeat et al., 2004) and in *M. japonicus* when fed dietary fucoidan for 8 weeks (Traifalgar et al., 2010).

The method of administering immunostimulants to prawns is important, as prawns are "grazers" and they do not eat their food whole, therefore, pelleted feeds need to be water stable to reduce nutrient/immunostimulants leaching from the pellet. Furthermore, incorporating the immunostimulant into the pellet is common practice rather than applying directly onto the pellet surface which increases leaching into the water prior to consumption (Smith et al., 2003).

The correct feed dose and timing is important when administering immunostimulants, which can be difficult without knowing the toxicity, potency or efficacy of the product being used (Sung et

al., 1994; Lorenzon et al., 1999). By assessing changes in the digestive gland and digestive tract structures using histology, it is possible to identify potential negative effects of ingredients used in feeds for prawns and teleost fish. In crustaceans the digestive gland (hepatopancreas) consists of blind-ending tubules containing four cell types F-cells (fibrillar); R-cells (restozellen); B-cells (blister-like) and E-cells (embryonic) (Vogt et al., 1985). In prawns, R-cells are responsible for lipoprotein metabolism and proliferation of R-cells can decrease rapidly, within 2 weeks after using feeds containing anti-nutritional factors (ANFs) (Piedad-Pascual et al., 1983; Vogt et al., 1985; Genc et al., 2007; Kumaraguru vasagam et al., 2007). Structural changes within liver cells, proximal and distal intestine of fish from ANFs have been documented in finfish within as little as 8 days (Storch et al., 1984: van den Ingh et al., 1991; Russell et al., 2001; Uran et al., 2008) and are commonly used to assess tissue responses to plant ingredients in aqua-feeds.

1.6.1 Fucoidan

Fucoidan is a sulphated polysaccharide from brown macro-algae (Class: *Phaeophyceae*) and contains the biologically active monosaccharide L-fucose. It has been demonstrated that fucoidan has anti-viral properties, by inhibiting enveloped DNA and RNA human viruses, such as; Herpes Simplex Virus (HSV) and Human Immunodeficiency Virus (HIV) (Baba et al., 1988). Fucoidan inclusion in prawn feeds increased disease resistance in *Marsupenaeus japonicus* (Takahashi et al., 1998) and *P. monodon* (Chotigeat et al., 2004; Immanuel et al., 2012) challenged with WSSV and increased immune response in *M. japonicus* (Traifalgar et al., 2010) and *P. monodon* (Felix et al., 2004; Immanuel et al., 2012).

The mechanism of viral inhibition by fucoidans is thought to be similar to that observed in humans (De Somer et al., 1968; Takahashi et al., 1998). *In-vitro*, fucoidan inhibited viral adsorption (HIV-1) into host cells (human T-cell) demonstrating potent anti-viral properties (Baba et al., 1988). In prawns, routine disease challenge models deliver the viral inoculum (WSSV) by intramuscular injection (IM) (Escobedo-Bonilla et al., 2006). However, this technique for delivering viral inoculum bypasses natural infection routes where healthy prawns commonly become infected by feeding upon diseased prawns (Alday-Sanz et al., 2002; Aguirre-Guzman et al., 2010; Sirikharin et al., 2015). In human studies, fucoidan blocks viral adsorption within the gut, which may be bypassed by using the IM technique. Other viral inoculation methods include oral intubation. However, in prawns, oral administration of an inoculum can be hindered through the foregut to the midgut, by hard mandibles and sharp angles after the oesophagus, resulting in a propensity to regurgitate food. However, anal intubation or reverse gavage (RG) has been proven to inoculate prawns by pipetting a consistent dose of inoculum through the anus and hindgut to the midgut with positive infection and 100% mortality in *L. vannamei* with necrotizing hepatopancreatic bacterium (Aranguren et al., 2010).

1.6.2 β-glucan

β-glucans are polysaccharides containing a structural glucose component linked by β-glycosidic bonds and are derived from the cell wall of many terrestrial and marine plants and yeasts (Meena et al., 2013). In fish and crustaceans, β-glucans are recognised by pattern recognition receptors and toll-like receptors within the innate immune system, and detect the pathogen-associated molecular patterns of the β-glucan components (Soderhall et al., 1994; Medzhitov and Janeway.,

1997; Meena et al., 2013). These components (β-glucan binding protein) activate immune functions such as encapsulation, coagulation, melanisation, and phagocytosis, which enhance the degranulation of granular haemocytes and activation of the proPO system (Vargas-Albores and Yepiz-Plascencia, 2000). β-glucan in feeds has shown to increase disease resistance and immune response to WSSV in *P. monodon* (Chang et al., 2003), *L. vannamei* (Bai et al., 2014) and *M. japonicus* (Itami et al., 1998).

1.6.3 Peptidoglycan

Peptidoglycan is a hetero-polymer containing cross-linked glycan strands and is a component of the cell wall of Gram positive bacteria (Schleifer and Kandler, 1972). Peptidoglycan has demonstrated to be a potent immunostimulant in aquaculture, derived from non-pathogenic bacteria such as *Bifidobacterium thermophilum* or *Brevibacterium lactofermentum* (Itami et al., 1998; Rattanachai et al., 2005; Purivirojkul et al., 2006). Peptidoglycan in prawn feeds increased disease resistance in *M. japonicus* when challenged with *Vibrio penaeicida* and WSSV (Itami et al., 1998), and increased PO activity, superoxide anion and bactericidal activity in *P. monodon* (Purivirojkul et al., 2006). Within 24 hours of feeding *M. japonicus* peptidoglycan, increased gene expression and subsequent crustin, lysozyme and antibacterial peptides involved in immune response were observed (Fagutao et al., 2008).

1.7 Aims of the study

There are many biotic and abiotic factors involved during commercial production of *P. monodon* which influence growth performance, immune response, aerobic metabolism and disease

resistance. This research has been conducted using an applied approach and has focused on commercially relevant inclusion levels and published inclusion levels. Dietary fucoidan has shown to be beneficial to penaeid prawns and the approach in this study has explored different applications: to environmental challenge, to low-level viral challenge indicative of GAV, to extreme viral WSSV challenge at a whole animal performance level (growth, FER and survival), and at a cellular then metabolic level to further assess benefits within a commercial application.

Several experiments have been conducted:

- To determine the potential of immunostimulants including fucoidans to affect different aspects of performance at commercially relevant inclusion levels.
- To determine the effects of different stressors encountered during commercial production on a range of performance and immune functions.
- To establish applied and fundamental information on the potential for nutritional mitigation for viral pathogens for the Australian prawn industry.

1.8 Notes on this study

The experimental chapters in this thesis have been prepared for publication in peer-reviewed journals. Therefore, some content may be repeated, in particular in the introduction, and within the materials and methods sections. Accordingly, research chapters contain abstracts, while general chapters do not.

Table 1.1. Examples of immunostimulants used on commercial prawn species and immune responses measured.

Immunostimulant	Species	Immune response	Author (s)
B-glucan			
β-1,3-glucan (*Schizophyllum commune*)	*P. monodon*	PA, PO, SOD, RB, THC	Chang et al., 2003
β-1,3,1,6- glucan (*Saccharomyces cerevisiae*	*P. monodon*	RB, CA	Song and Hsieh, 1994
β-1,3-Glucan *(S. cerevisiae)*	*P. monodon*	HA	Pais et al., 2008
β-1,6-glucan *(S. cerevisiae)*	*L.vannamei*	THC, SOD, HP	Campa-Cordova et al., 2002
β-glucan (*S. cerevisiae*), glycyrrhizin	*L.vannamei*	THC, PO, RB, SOD	Chang et al., 2011
β-glucan (*S. cerevisiae*)	*F. chinensis*	THC, proPO	Bae et al., 2012
Yeast glucan	*P. monodon*	PO	Sung et al., 1994
Glucan *(Acremonium diospyri)*	*F. indicus*	THC, proPO, ROI,	Anas et al., 2009
Candida sake (marine yeast S165)	*F. indicus*	THC, PO, RB	Sajeevan et al., 2009
Candida aquaetextoris (S527)	*P. monodon*	proPO, THC, AMPs	Babu et al., 2013
Debaryomyces hansenii, Candida tropicalis	*F. indicus*	THC, PO, RB	Sarlin and Philip, 2011
mannan oligosaccharides	*P. semisulcatus*	not measured	Genc et al., 2007
Fucoidan/Algal polysaccharides			
Sargassum duplicatum	*L. vannamei*	THC, PO, RB	Yeh et al., 2006
Cynodon dactylon extract	*P. monodon*	not measured	Balasubramian et al., 2007
Momordica charantia extract	*P. monodon*	not measured	Balasubramian et al., 2007
Ergosan (1% alginic acid)	*L. vannamei*	THC, BA	Montero-Rocha et al., 2006
Fucoidan *(Undaria pinnatifida)*	*M. japonicus*	THC, PO, SAA	Traifalgar et al., 2010
Fucoidan (*Cladosiphon okamuranus*)	*P japonicus*	not measured	Takahashi et al., 1998
Fucoidan *(S. polycytum)*	*P. monodon*	SAA, PA,	Chotigeat et al., 2004
Fucoidan *(Cyanothece sp.)*	*L.vannamei*	THC, SOD, HP,	Campa-Cordova et al., 2002
Sargassum spp.	*P. monodon*	not measured	Immanuel et al., 2010
Sargassum wightii	*P. monodon*	THC, PO, RB, SOD, PA	Immanuel et al., 2012
Sargassum wightii	*P. monodon*	proPO	Felix et al., 2004
red seaweed *(Gracilaria tenuistipitata)*	*L.vannamei*	THC, PO, RB, SOD, PA, BCA	Hou and Chen, 2005
Sodium alginate	*P. monodon*	THC, PO, proPO, RB, SOD	Liu et al., 2006

continued

Gracilaria lemaneiformis	*L. vannamei*	SOD	Yu et al., 2016
Undaria pinnatifida	*P. monodon*	SOD, PO,	Niu et al., 2015
Peptidoglycan/Probiotics			
AquaVac™Vibromax™	*P. monodon, L. vannamei*	not measured	Wongtavatchai et al., 2010
Peptidoglycan	*P. monodon*	PO, RB, BA	Purivirojkul et al., 2006
Peptidoglycan (*Bifidobacterium thermophilum*)	*M. japonicus*	PA	Itami et al., 1998
Bacillus S11	*P. monodon*	PA, PO, BA	Rengpipat et al., 2000
Pseudomonas MCCB 102,103	*P. monodon*	BA	Preetha et al., 2010
Bacillus MCCB 101, *Micrococcus* MCCB 104	*P. monodon*	AMPs	Antony et al., 2011
Terrestrial plant extract			
Dunaliella extract	*P. monodon*	THC, PO, BCA	Supamattaya et al., 2005
Padina boergesenii	*F. indicus*	THC, PA, BA	Ghaednia et al., 2010
licorice extract	*L vannamei*	THC, PO, SOD, NOS	Chen et al., 2010
Glycyrrhizin and β-glucan(*S. cerevisiae*)	*L. vannamei*	THC. PO, SOD, RB	Chang et al., 2011

PA = phagocytic activity, HAR = haemocyte adherence rate, HA = haemagglutinins, RB = respiratory burst, THC = total haemocyte count, PO = phenoloxidase activity, proPO = prophenoloxidase activity, SOD = superoxide dismutase, ROI = reactive oxygen intermediates, BA = bactericidal activity, HP = haemocyte protein, SAA = serum antibacterial activity, BCA = bacterial clearance activity, NOS = nitric oxide synthase activity, CA = chemilumunescence assay and AMPs = antimicrobial peptides.

1.9 References

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Preliminary screening of fucoidan in *Penaeus monodon* feeds; effects on growth performance and immune response

CHAPTER II

2.1 Abstract

The use of oral immunostimulants in prawn feeds is increasing worldwide to improve survival of prawns to diseases caused by viruses and bacteria. This study determined whether commercial fucoidan ingredients extracted individually from *Undaria pinnatifida* (UP85 and F50), *Fucus vesiculosus* (FV90) and *Ascophyllum nodosum* (MMB) enhanced the THC, DHC and PO activity of prawns, and whether fucoidan caused changes in digestive gland structure after 15 d. Five isonitrogenous, isolipidic and isoenergetic feeds were formulated to contain 44% crude protein, 7% crude lipid and 15.9 MJ kg^{-1} of gross energy. A basal control feed was formulated as per fucoidan feeds, however without fucoidan and a commercial prawn feed (Ridley Aqua-Feeds, Prawn Starter Enhance) was used as a reference feed treatment. Six *P. monodon* (PL 60, mean weight 1.247 ± 0.016 g) were stocked haphazardly into each tank of a 24 tank experimental system. Six feeds in quadruplicate were randomly assigned to tanks. Prawns were fed experimental feeds twice daily (09:00 and 18:00) to satiation. At the conclusion of the experiment the mean final weight (4.004 ± 0.070 g) and body weight gain (1.979 ± 0.068 g) of prawns were not different between treatments (P = 0.828 and 0.857 respectively). Mean feed intake (FI) was similar between treatments ranging from 0.98 g DM Pd^{-1} for prawns fed the commercial feed up to 1.23 g DM Pd^{-1} when fed the basal feed. The feed efficiency ratios (FER) of prawns fed all treatments were not different, the control group had the lowest FER at 59.98% and the commercial group had 75.67%. Specific growth rate (SGR) ranged from 4.34% d^{-1} for prawns fed the basal treatment up to 4.79% d^{-1} when fed the marine macro-algae blend meal treatment (P = 0.885). Mean survival was similar across all treatments and ranged from 70.83 – 95.83%. Total haemocyte count (THC) ranged from 3.42 ± 0.31 cells x 10^7 ml^{-1} (control group) to 4.02 ± 0.26 cells x 10^7 ml^{-1} (P = 0.475) for prawns fed immunostimulants. However, when haemocytes were quantified by granulocytes per ml^{-1}, prawns fed the FV90 feed treatment had a significantly higher number of

granulocytes ml^{-1} (1.72 ± 0.25 cells x 10^7 ml^{-1}) compared to the prawns fed F50 (1.02 ± 0.11 cells x 10^7 ml^{-1}), although similar to the prawns fed all other feeds. The prawns fed the basal feed had significantly higher (P = 0.001) PO activity (0.028 ± <0.01 Δ Abs/min^{-1}) followed by the prawns fed on MMB (0.018 ± <0.01 Δ Abs/min^{-1}) and were comparable to prawns fed FV90 (0.016 ± <0.01 Δ Abs/min^{-1}), and all other treatments. The digestive gland (DG) tubules in all prawns from all treatments presented as tightly packed tubules contained within the inter-connective tissue with star-shaped tubule lumens, indicative of healthy animals. This study showed that feeding *P. monodon* feeds containing fucoidan had no negative impacts on growth rates, immune response or digestive gland health. The increased number of granular haemocytes in prawns fed FV90 is of great importance, as small granular haemocytes (SGH) and large granular haemocytes (LGH) play important roles in the defence mechanism of crustaceans. Future research should determine if higher fucoidan inclusion levels of fucoidan could promote growth and further enhance immune responses of *P. monodon*.

2.2 Introduction

The black tiger prawn, *Penaeus monodon* is a commercially important species and is the main penaeid prawn species cultured in Australia (FAO, 2012). Total prawn production in Australia during 2011-12 was 2900 tonnes valued at $59 million (ABARES, 2013). In Australia the most important viral disease in prawn aquaculture is Gill-Associated Virus (GAV), an RNA Nidovirus endemic to Queensland that causes sporadic mortality in commercial prawn farming (Spann et al., 1997; Walker et al., 2001). GAV presents similarly to Yellow Head Virus, by affecting the lymphoid organ tubule structure and digestive gland, however there is no yellowing of the carapace and mortality of experimentally infected animals can reach 100% within 3 days (Spann et al., 2003). Prawns produced in Queensland are thought to be chronically infected with GAV; however these prawns exhibit no clinical signs of disease and the impact on production is minimal (Cowley et al., 2000; de la Vega et al., 2007). Suboptimal culture conditions, such as a significant decrease in water temperature and salinity, causes a disruption in homeostasis inducing a stress response that can initiate an acute GAV infection resulting in a significant loss of prawns and decreased production (de la Vega et al., 2004; Liu et al., 2006).

Prawns are vulnerable to invading microorganisms entering the animal via wounds in the exoskeleton or with food particles into the stomach initiating an immune response (Bauchau, 1981). Haemocytes (hyaline cells, small granular and large granular cells) are the first point of defence against pathogens and are located in the haemolymph (Bauchau, 1981; Smith et al., 2003).

Fucoidan has been reported to increase total haemocyte counts (THC), phenoloxidase activity (PO activity) in *Marsupenaeus japonicus* (Traifalgar et al., 2010) and survival in *M. japonicus* and *P. monodon* challenged with WSSV (Takahashi et al., 1998; Chotigeat et al., 2004; Immanuel et al., 2012). The mechanism of how fucoidan achieves viral inhibition is

thought to be similar to that observed in humans (De Somer et al., 1968; Baba et al., 1988), In these cases, the fucoidan demonstrated potent antiviral properties, including inhibition of viral adsorption (HIV-1) onto host cells (human T-cell) *in vitro* (Baba et al., 1988). In prawns, WSSV inhibition in *M. japonicus* was achieved when partially purified fucoidan from *Cladosiphon okamuranus* was administered in feeds for 15 days prior to challenging prawns with WSSV (Takahashi et al., 1998). The mechanism in prawns is thought to be the same as in humans; however Baba et al. (1988) also stated that further investigations were required to demonstrate how fucoidan restricts viral adsorption within the digestive tract.

The importance of timing the administration of immunostimulants to prawns is critical, as immunostimulants need to be administered prior a disease outbreak, due to the time required to initiate an immune response (Raa, 2001). Time required to achieve this has been recorded up to 24 and 30 days respectively for *P. monodon* fed β-1,3-glucan and peptidoglycan (Chang et al., 2000; Purivirojkul et al., 2006), while increased immunity was achieved in *P. monodon* within 15 d fed crude fucoidan (Chotigeat et al., 2004) and in *M. japonicus* when fed dietary fucoidan for 8 weeks (Traifalgar et al., 2010). It is also commonly agreed that fucoidan may not be economically sustainable for the entire production duration (Takahashi et al., 1998). The method of administering immunostimulants to prawns is important, as prawns are "grazers" and they do not eat their food immediately, therefore, the need for feeds to be water stable is very important to reduce nutrient/immunostimulants leaching from the pellet.

The potential toxic effects of immunostimulants in aqua-feeds can be assessed by conducting histopathology of the digestive gland (hepatopancreas) (Vogt et al., 1985; Tacon, 1997; Genc et al., 2007; Kumaraguru vasagam et al., 2007). Digestive gland cellular structure can change within 14 d after feeding prawns feeds containing anti-nutritional factors (ANFs) or toxic substances (Piedad-Pascual et al., 1983; Vogt et al., 1985; Genc et al., 2007). Therefore, digestive glands can be assessed as an indicator organ for monitoring the health and nutrition

of prawns when screening novel immunostimulants and ingredients which have undergone chemical extrusion, purification or contain toxic substances.

The aims of this study were to:

- To conduct preliminary screening of sulfated fucoidan in *P. monodon* feeds from different species of brown macroalgae (*Undaria pinnatifida*, *Fucus vesiculosus* and *Ascophyllum nodosum*) to determine their potential effects on growth performance and survival.
- To measure haemocyte mediated immune responses (THC, DHC and PO) of *P. monodon* after feeding on feeds containing fucoidan.
- To describe the histology of the digestive gland of *P. monodon* after feeding for 15 d.

2.3 Materials and methods

2.3.1 Prawn stock

Twelve day old black tiger prawn (*P. monodon*) postlarvae (PL) were obtained from Australian Prawn Farms, Ilbilbie, Queensland and were the progeny of wild Northern Territory broodstock. Postlarvae were transported by air to the University of Tasmania, Launceston, Tasmania where they were held under quarantine (Special Authority Number, SA 11-09). Approximately 10,000 PL were on-grown to 2 g, in two 1000 L tanks within a recirculating seawater system. Postlarvae were fed a mixture of live *Artemia spp.* enriched with *Pavlova lutheri* and commercial prawn crumble (Ridley Aqua-Feed, Prawn Starter Enhance) twice daily to satiation until PL 28, and thereafter were fed to satiation twice daily on a commercial prawn feed. During on-growing PL were maintained under optimum environmental conditions (salinity: 30-32‰, photoperiod: 12:12 light dark cycle, temperature: 28-30°C) and water quality parameters (NH_3: <1 ppm, NO_2: <1 ppm, NO_3: <10 ppm, pH: 8.2) were measured using a saltwater test kit (API™).

2.3.2 Experimental system

A recirculating seawater system comprising of twenty four 45 L rectangular (surface area = 0.24 m²), blue polyethylene tanks were used. Water quality was maintained by biofiltration, UV sterilization (18 W Emperor Aquatics) and solids removal. Water was heated by an 8.1 kW reverse cycle air conditioner (Mitsubishi Electric, MUZ-GA71VA) and a submerged water heater (Aqua One, 60 W) provided additional heating. Aeration was provided by individual air-stones in each tank and within the sump. Tanks were fitted with clear polycarbonate hinged tank covers to stop prawns escaping, and tank outlets were screened with fibreglass flyscreens (1 mm²) to keep prawns within tanks. The average flow rate was 3.3 L/min, exchanging each tank water volume every 13.6 min.

2.3.3 Feed formulation

The basal feed was formulated at 44% crude protein, 7% crude lipid and 15.9 MJ kg^{-1} of gross energy, to be comparable to commercially available feeds (Ridley Aqua-Feed, Prawn Starter Enhance) containing commercially relevant ingredients used within the aqua-feed industry (Table 2.1). The basal feed (control) was formulated to contain 33 $g.kg^{-1}$ α-cellulose which was completely or partially substituted with the individual test ingredient. Prawn Starter Enhance (Ridley Aqua-Feed) was included as an additional reference feed treatment to compare against the basal feed treatment, and between experiments. The Prawn Starter Enhance feed was supplied as a mash and was pelleted at the University of Tasmania using a commercial pasta maker (ItalPast, Mac 10s) and feeds were pelleted using the same equipment.

Table 2.1. Ingredient inclusion as is ($g.kg^{-1}$) and mean chemical composition (n=2) of experimental feeds ($g.kg^{-1}$ DM) +/- S.E.

	Feeds				
Ingredients (as is $g.kg^{-1}$)	**BASAL**	**UP85**	**FV90**	**F50**	**MMB**
Fishmeal	500.00	500.00	500.00	500.00	500.00
Wheat flour	298.70	298.70	298.70	298.70	298.70
Krill meal	50.00	50.00	50.00	50.00	50.00
Wheat gluten	70.00	70.00	70.00	70.00	70.00
Test ingredient	0.00	0.20	0.20	0.20	33.00
α-cellulose	33.00	32.80	32.80	32.80	0.00
Macro mineral mix[1]	33.00	33.00	33.00	33.00	33.00
Lecithin	10.00	10.00	10.00	10.00	10.00
Vitamins & micro mineral mix[2]	2.00	2.00	2.00	2.00	2.00
Cholesterol	1.00	1.00	1.00	1.00	1.00
Stay C	1.00	1.00	1.00	1.00	1.00
Pigment	0.50	0.50	0.50	0.50	0.50
Banox E	0.20	0.20	0.20	0.20	0.20
Choline chloride	0.60	0.60	0.60	0.60	0.60
Proximate composition $g.kg^{-1}$					
Dry matter	911.30 ± 0.13	908.93 ± 1.02	900.06 ± 0.35	916.68 ± 2.60	912.17 ± 2.52
Crude protein	479.69 ± 3.85	476.50 ± 4.60	467.73 ± 6.12	473.36 ± 4.32	476.42 ± 4.66
Crude lipid	97.19 ± 1.54	98.42 ± 4.99	97.95 ± 0.89	99.68 ± 1.17	96.54 ± 0.06
Gross energy ($MJ.kg^{-1}$)	18.53 ± 0.02	18.35 ± 0.07	18.56 ± 0.03	18.41 ± 0.01	18.37 ± 0.05
Ash	72.66 ± 0.18	71.37 ± 0.36	72.54 ± 0.10	71.09 ± 0.40	74.42 ± 0.76
Protein:energy (g CP.MJ GE^{-1})	25.89	25.97	25.20	25.71	25.93

Feeds contain: BASAL = Control, UP85 = Maritech® *U. pinnatifida*, FV90 = Maritech® *Fucus vesiculosus*, F50 = Fucorich® *U. pinnatifida* extract, MMB = marine macro-algae blend meal (AF1/07, 50:50 *U. pinnatifida* & *A. nodosum*).

[1]Macro mineral mix (Davis and Lawrence, 1997), (g.kg): K_2PO_4, 10; $Ca(H_2PO_4)_2$, 10; NaH2PO4, 10; $MgSO_4.7H_2O$, 0.3.

[2]Vitamin and micro mineral premix (Conklin, 1997; Davis and Lawrence, 1997). Vitamins (mg.kg): thiamine-HCl, 60; riboflavin, 25; nicotinic acid, 40; pyridoxine-HCl, 50; pantothenic acid, 75; biotin, 1; folic acid, 10; vitamin B12, 0.2; myo-inositol, 400; L-ascorbic acid, 200; vitamin A acetate, 10; DL-α-tocopherol acetate, 100; vitamin D3, 0.1; menadone sodium bisulfite, 5, α-cellulose, 823.7. Micro minerals (mg.kg): $CuSO_4.5H_2O$, 32; $ZnSO_4.7H_2O$, 15; Na_2SeO_3, 0.2; α-cellulose, 52.

2.3.4 Test ingredients

Feeds containing purified marine macro-algal test ingredients (Maritech® *U. pinnatifida*, Maritech® *Fucus vesiculosus* and Fucorich® *U. pinnatifida* extract) were formulated to contain 0.2 g.kg^{-1} (Chotigeat et al., 2004) test ingredient 'as is', while marine macro-algae blend meal (*Undaria pinnatifida* & *Ascophyllum nodosum*, AF01/07) was formulated at 33 g.kg^{-1} (Table 2.1).

Table 2.2. Individual test ingredients and corresponding fucoidan concentration.

Test ingredients	Fucoidan component	Macro-algal source
Marine macro-algae blend meal (AF1/07)[1]	4% fucoidan	50:50 *U. pinnatifida* & *A. nodosum*
Maritech® *Undaria pinnatifida*[1]	90.2% fucoidan	*U. pinnatifida*
Maritech® *Fucus vesiculosus*[1]	92.6% fucoidan	*F. vesiculosus*
Fucorich® *Undaria pinnatifida*[1]	50% fucoidan	*U. pinnatifida*

[1]Ingredients supplied by Marinova Pty Ltd, Cambridge Tasmania, Australia.

Fish meal (Skretting Australia, Cambridge, Tasmania) and South American krill meal (Skretting Australia, Cambridge, Tasmania) were included as sources of protein and lipid, while krill meal was also a feed attractant and additional source of protein. Wheat flour (Four Roses, New South Wales, Australia) and wheat gluten (MP Biomedicals) were binders. Cholesterol (Sigma-Aldrich) was added at 1 g.kg^{-1} and soy lecithin (Lucas Meyer Eurologistic, IIIinois) at 10 g.kg^{-1}. Macro mineral premix was made from individual minerals (Sigma-Aldrich) and were included at 33 g.kg^{-1}, containing the recommended mineral concentrations for marine shrimp (Davis and Lawrence, 1997). Vitamin and micro mineral premix was made from individual vitamins and minerals (Sigma-Aldrich) and was added at 2 g.kg^{-1} according to the recommended vitamin and mineral concentrations for marine shrimp (Conklin, 1997; Davis and Lawrence, 1997) (Table 2.1).

Marine macro-algae was provided as coarse particles, approximately 750 µm, and was ground to a finer powder using a bench top hammer mill (Culatti micro hammer-cutter mill, MFC), while other test ingredients were supplied as fine powders. Fish meal was sifted through a 1 mm sieve (Greer and Ashburner, 1-C). Dry bulk ingredients were mixed in a mixer (Brice Australia, Pty Ltd, VFM-20C) for 20 min before small dry ingredients including test ingredients were homogenised in a subsample of the bulk mix, then returned to the bulk mix and mixed for further 20 min. Lecithin, choline chloride (dissolved in distilled water) and distilled water were added to the dry mixture, before further mixing for 20 mins until a crumbly dough could be formed. The dough was then passed twice through a 2 mm die of a commercial pasta maker (ItalPast, Mac 10s) and cut to approximately 5 mm lengths. Pellets were steamed for 5 min (Tefal, Steam Cuisine) before air dried (Forma Scientific, 68732-1) until feeds contained less than 10% moisture. Feeds were stored in airtight bags at -20°C until required.

2.3.5 Test ingredient validation

Test ingredients in the current study were included in feeds at low inclusions (0.2 - 33 $g.kg^{-1}$). An analysis of variance (ANOVA) was conducted for validating the homogeneity of the test ingredient in the feed by determining the optimum number of times each feed was pelleted. The optimum number of times pelleted was determined by substituting fucoidan for ytterbium (III) acetate (Yb) (Sigma-Aldrich), an inert maker that was included in feeds at a rate of 202 $mg.kg^{-1}$, using a modified basal feed, excluding vitamins, minerals, cholesterol, pigment, Stay-C and Banox-E. The modified basal feed containing Yb was passed through the pasta maker three times and samples of approximately 10 g were taken in triplicate (start, middle and end) for each pelleter run time, which was approximately 15 min. Samples were freeze-dried to a constant weight. Samples were pre-digested in 2 ml of concentrated HNO_3 overnight before the addition of a further 2 ml of HNO_3 before heating to 90°C for 3 h. Once digests were clear they were cooled and diluted volumetrically 100 times before analysing by Atomic Absorption Spectroscopy (AAS) with a N_2O-acetylene flame. The digest was compared to serial diluted Yb standards (EM Science).

2.3.6 Pellet water stability

Pellet water stability was measured in the experimental tanks under experimental conditions (without prawns) in triplicate using feed samples of approximately 2 g. Pellets were left in tanks for 1 h, before siphoning pellets onto a 250 µm screen and transferring them to a glass Petri dish. Samples were dried in a drying oven at 135°C for 4 h, cooled to room temperature and weighed. Water stability was calculated as the final dry matter (DM) as a percentage of the initial pellet DM. Pellet water stability data were used to correct the apparent feed intake (g DM), for DM loss over 1 h due to the majority of feed being consumed after 1 h of

offering feed (Cruz-Suarez et al., 2001). Apparent feed intake (FI g DM) was used to determine feed efficiency ratio (FER) and food conversion ratio (FCR).

2.4 Experiment period

2.4.1 Acclimation

Six *P. monodon* (PL 60, mean weight 1.247 ± 0.016 g) were stocked randomly into each of 24 tanks in the experimental system. The start of the acclimation and experimental periods were staggered over 2 d (12 tanks per day) to ensure sufficient time available during final sampling and to conduct laboratory analyses on fresh haemolymph. Prawns were fed on Prawn Starter Enhance (Ridley Aqua-Feeds) twice per day (09:00 and 18:00) to satiation for 7 d and feed intake was measured. Before feeding, all uneaten pellets, faeces and exuvia were siphoned from tanks. During the acclimation period any mortalities were replaced with new randomly selected prawns from the same stock population. Water quality was assessed daily (NH_3: <0.25 ppm; NO_2: <0.25 ppm; NO_3: <20 ppm; pH: 7.9-8.2) using a saltwater test kit (API™).

2.4.2 Experimental protocol

At the start of the experiment all prawns (PL 67) were blotted dry with paper towel and individually weighed (mean weight 2.025 ± 0.029 g) by placing prawns in a closed container on a balance (AND, FX300). Six feed treatments were randomly assigned to tanks (n = 4). Prawns were fed experimental feeds twice daily (09:00 and 18:00) to satiation, increasing from 29.5 – 32.0% $bw.d^{-1}$ over the 15 d experiment and feed intake was recorded. The feed portions were split 40:60 with the 60% portion of feed fed in the afternoon with feed remaining in tanks until 09:00 the next morning. Tanks were cleaned by siphoning waste into

a bucket, while uneaten feed was collected on a 250 µm screen. Uneaten feed was pooled per tank at stored at -20°C until the conclusion of the experiment before being dried at 135°C for 2 h, and used to calculate feed intake. The experiment was concluded after 15 d, pre-determined by previously published studies that achieved 100% increase in weight of similar sized animals and determined any toxic effects from the feeds (Vogt et al., 1985; Chotigeat et al., 2004; Felix et al., 2004). Three artificial hides made from bunched strips of fibreglass flyscreen material suspended below a block of polystyrene were added to each tank to increase survival of moulting animals. Water temperature and salinity ranged between 31-32.5°C and 31-33‰ respectively. Temperature was recorded hourly by a temperature data logger (HOBO® Pendant, UA-002-XX, Onset Computer Corporation, Pocasset, USA) while salinity was measured by daily using a salinity refractometer (IWAKI, TD5Q8).

2.4.3 Initial sampling

Prawns were moult staged according to Smith and Dall (1985) using a stereo microscope (Olympus SZ40). Sixteen randomly selected prawns (mean = 3.306 ± 0.196 g) in intermoult (stage C) were sampled on day 1 of the experiment; from a stock holding tank for immune parameter analyses: phenoloxidase activity (PO activity), total haemocyte counts (THC) and differential haemocyte counts (DHC), granular haemocyte counts (GHC) and histology. Approximately 80 µl of haemolymph was withdrawn from the cardiac sinus of each prawn using a sterile 25 G needle and 1.0 ml syringe pre-rinsed with anticoagulant (shrimp salt solution (SSS); 450 mM NaCl, 10 mM KCl, 10 mM EDTA.NA_2, 10 mM HEPES, pH 7.3, 850 mOsm kg^{-1}) (Vargas-Albores et al., 1993) and placed into a 2.0 ml micro-centrifuge tube. Immediately, 25 µl of haemolymph was removed into another 2.0 ml micro-centrifuge tube containing 25 µl of 10% formalin buffered with 0.45 mM NaCl for DHC and THC, while 50 µl of pre-cooled SSS (4°C) was mixed with 25 µl of haemolymph in an additional

micro-centrifuge tube for PO activity. The haemolymph samples were placed on ice and analysed on the same day.

2.4.4 Immune response parameters

2.4.4.1 Differential haemocyte count (DHC)

Formalin fixed haemolymph was used for DHC according to Sritunyalucksana et al. (2005). After allowing haemocytes to fix in formalin for approximately 20 min, 50 µl of Rose Bengal solution (Sigma-Aldrich, 1.2% Rose Bengal in 50% ethanol) was added to the micro-centrifuge tube and incubated at room temperature for 20 min (Sritunyalucksana et al., 2005). Stained haemolymph samples from four prawns were pooled together and triplicate blood smears for each pooled sample were made using one drop (20 µl) of stained haemolymph solution smeared onto a clean microscope slide. Smears were air-dried, then counterstained with haematoxylin (Sigma-Aldrich) for 3 min, before rinsing in tap water for 2 min followed by immersion for 1 min in 95% iso-propyl alcohol and 2 min in 100% iso-propyl alcohol (repeated twice), using an automated diversified stainer (Sakura, DRS-60) (Sritunyalucksana et al., 2005). Following dehydration, slides were placed in xylene for 2 min and mounted with a cover-slip using DPX mountant (Sigma-Aldrich). Granulocyte ratios (DHC) were determined by counting 200 haemocytes per smear under 400 x objective of a light microscope (Olympus BH-2) and expressed as a proportion of granulocytes (GHC) (small-granular and large-granular haemocytes) in 200 total haemocytes, i.e. count/200 x THC (Sritunyalucksana et al., 2005).

2.4.4.2 Total haemocyte count (THC)

One drop of Rose Bengal stained pooled haemolymph mixture from DHC samples was placed on a Neubauer Improved Bright-line haemocytometer under 400 x magnification of a light microscope (Olympus, CH30). At least 200 haemocytes were counted in triplicate for quantifying the total number of haemocytes per ml^{-1} of haemolymph.

2.4.4.3 Phenoloxidase (PO) activity

The PO activity method followed the protocol from Hernandez-Lopez et al. (1996) using the modified 96 well micro-plate method. Haemolymph diluted in SSS was centrifuged (Eppendorf, S415D) at 600 x *g* at 4°C and the supernatant was removed. The cell pellet was rinsed with 200 µl cacodylate buffer (10 mM sodium cacodylate, 10 mM $CaCl_2$, pH 7.0) before centrifuging again, supernatant was removed once more and haemocytes suspended in 200 µl of cacodylate buffer. Haemocyte cell suspensions from the same four prawns were pooled according to the protocol for DHC and THC.

PO activity was measured spectrophotometrically (in triplicate) by the formation of dopachrome from the substrate, L-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich). Fifty microliters of pooled haemocyte cell suspension was incubated with 50 µl of laminarin (Sigma-Aldrich) (1 mg/ml in cacodylate buffer) in a flat-bottomed 96 well micro-plate (TTP®, 92696) for 30 min at 25°C. Fifty microliters of L-DOPA (3 mg/ml in cacodylate buffer) was added to each well and incubated at 25°C for 10 min, before reading the optical density at 492 nm on an ELISA reader (SPECTRA, Rainbow Thermo). Enzyme activity was expressed as the change in absorbance per min (Δ $Abs.min^{-1}$) per 100 µl of haemolymph.

2.4.5 Digestive gland histopathology

The digestive gland and cephalothorax of each prawn were injected with approximately 50 µl freshwater Davidson's fixative (300 ml 95% ethanol, 200 ml 37% formaldehyde, 100 ml glacial acetic acid, 300 ml distilled water) immediately after removing haemolymph. The cuticle was then cut dorsally from anterior to posterior to allow the fixative to penetrate the prawn. The cephalothorax was transversely dissected from the abdomen and the cephalothorax was immersed in freshwater Davidson's fixative (Bell and Lightner, 1988). Twenty four hours later the samples were transferred to 70% ethanol, where they were stored until processing. Prior to processing, eyes and all appendages were removed from the cephalothorax; the two sections were placed cut-side down in a cassette (Bell and Lightner, 1988). Samples were dehydrated in a graded series of ethanol and infiltrated with paraffin wax before they were processed using a Sakura Finetechnical Co. LTD (model, 4634) tissue processor and embedded in paraffin wax (Shandon Histocentre 3). Blocks were sectioned at 5 µm on a microtome (Microm, HM340), sections were placed on glass slides and dried at 37°C (Ratek Instruments) for approximately 12 h. Sections were stained with haematoxylin and eosin (H&E, Sigma) and examined at 200 and 400 x magnification using a light microscope (Olympus BH-2) fitted with a digital camera (Leica, DC300F). Transverse image of digestive glands were captured using Leica IM50 software, version 1.20. Transverse sections of digestive gland tubules in the proximal region were assessed for histopathology.

2.4.6 Final sampling

The experiment concluded after 15 d and prawns were individually weighed for calculating growth rates. The total number of prawns remaining in each tank was counted and used to calculate survival. Prawns were moult staged and four animals per tank in the intermoult stage were pooled for haemolymph assays (DHC, THC and PO activity) and digestive gland

(DG) histology as per sections 2.3.4.5 to 2.3.4.8. If fewer than 4 animals per tank were in intermoult stage, twice the volume of haemolymph from one intermoult animal was removed to retain the same final volume of haemolymph required for analyses.

2.4.7 Chemical analyses

Experimental feeds were freeze-dried (Dynavac, FD3) to a constant weight, and chemical composition of feeds was analysed in duplicate at the Nutrition Laboratory (University of Tasmania, Launceston, Tasmania). Feeds were homogenised by hand to a fine powder using a mortar and pestle, before being wrapped in aluminium foil and placed in an airtight plastic bag at -20°C until required.

Crude protein analysis was conducted using Kjeldahl (FOSS Kjeltec™ 8100, crude protein was calculated as N x 6.25), crude lipid (Bligh and Dyer, 1959), gross energy by bomb calorimetry (Gallenkamp Autobomb, CAB101), ash by combustion (SEM 102C muffle furnace) at 600°C for 2 h, using standard laboratory methods in accordance with AOAC (1995). Moisture content of feeds was determined for FI and conducted by drying a 2 g sample of feed in triplicate at 135°C to a constant weight, moisture content was calculated by the weight loss.

2.4.8 Statistical analyses

Tanks were considered a replicate unit for growth and survival data, while four prawns per tank were pooled for average immune responses. Homogeneity and normality of data was assessed using Levene's test for equality and residual plots. The means of small ingredient inclusion validation, growth, immune parameters and pellet water stability were compared using a one-way Analysis of Variance (ANOVA) in IBM SPSS STATISTICS (version 19)

for comparison, significant results were considered at $P < 0.05$ using a Tukey's HSD test. Survival percentage data were arcsine transformed in IBM SPSS STATISTICS (version 19). Initial baseline data for THC, DHC, GHC and PO were not included in statistical tests and were used as a reference, due to prawns being held in a different recirculation system and at higher stocking densities. Data were presented as mean ± standard error with the exception of small ingredient inclusion figure where data were stated as means ± standard deviation. Tables and figures were constructed using Microsoft Office Excel 2007 and Sigma Plot, version 10.0 (Systat Software, Inc).

2.4.9 Calculations

Prawn days (Pd) was calculated as:

$$\text{Pd (mean prawn.days}^{-1}) = (d._{\text{feeding}} / d._{\text{experiment}})$$

Where: $d._{\text{feeding}}$ = cumulative number of days where prawns were alive and feeding, $D._{\text{experiment}}$ = duration of experiment in days. Pd is the mean number of surviving prawns per day for calculating daily FI and FER to standardise data due to mortality from predation.

Water stability was calculated as:

$$\text{Water stability (\%)} = (FW_{dry}\ (g) / (IW_{wet}\ (g) \times \%DM / 100)) \times 100$$

Where: FW_{dry} = final dry weight after immersion, IW_{wet} = initial weight of pellet before drying and %DM = percentage dry matter in pellets.

Feed intake (FI) (g DM^{-1} Pd^{-1}) was calculated as total feed consumed (g dry matter) less uneaten feed and dry matter losses due to leaching.

$$FI = (\text{consumed } W_{\text{feed DM}}\ (g) - M_{loss}) \times W_{stability} / 100) \times Pd^{-1}$$

Where: $W_{\text{feed DM}}$ = total dry weight of feed consumed (g), M_{loss} = pellet moisture loss (g), $W_{stability}$ = pellet dry matter loss, Pd = prawn.$days^{-1}$ where mean number of surviving prawns per day.

Weight gain was calculated as:

Weight gain (g) = (FW_{wet} (g) – IW_{wet} (g))

Where: FW_{wet} = final wet weight, IW_{wet} = initial wet weight.

Specific growth rate (SGR) was calculated as:

SGR (% d^{-1}) = ((Ln W_{final} – Ln$W_{initial}$)) / d. x 100

Where: W_{final} = mean final wet weight (g), $W_{initial}$ = mean initial wet weight (g), d. = no. of days (Ricker, 1979).

Average weekly gain (AWG) was calculated as:

AWG = (W_{final} (g) – $W_{initial}$ (g)) / wk

Where: W_{final} = mean final wet weight (g), $W_{initial}$ = mean initial wet weight (g), wk = duration of experiment in weeks.

Feed efficiency ratio (FER) was calculated as:

FER = (W_{final} (g) / FI (g)) x 100)

Where: W_{final} = mean wet weight gain (g), FI = feed intake (g DM mean Pd^{-1}) after adjustments for uneaten feed and leaching. Mean wet weight was used over total tank weight gain due to 50% mortality rate in some tanks; therefore these tanks had a negative total weight gain.

Total haemocyte count (THC cells.ml^{-1}) was calculated as:

THC = (MHC x 1 x 10^4) x DF

Where: MHC = mean number of haemocytes per large square of haemocytometer, 1 x 10^4 = conversion factor, changing millimetres to millilitres, DF = dilution factor of the addition of fixative and stain (Sritunyalucksana et al., 2005).

Differential haemocyte count (DHC %) was calculated as:

DHC = (GH / haemocytes counted) x 100

Where: GH = total number of small and large granular haemocytes.

Granular haemocyte count (GHC cells.ml^{-1}) was calculated as:

GHC = (DHC x THC)

Where: DHC = mean number of small and large granular haemocytes as a percentage, THC = mean number of haemocytes per ml of haemolymph (Sritunyalucksana et al., 2005).

2.5 Results

2.5.1 Small ingredient inclusion validation

There was no significant effect of the numbers of times the dough was pelleted on the measured ytterbium concentration (P = 0.386) (Figure 2.1). The measured feed samples (n =3) from each pelleting time was within the formulated inclusion of ytterbium (202 mg.kg^{-1}) (Figure 2.1).

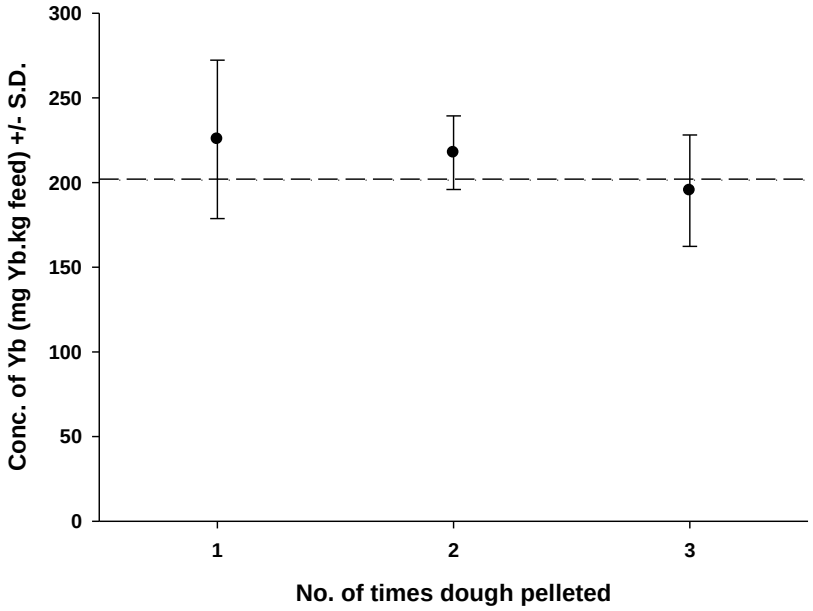

Figure 2.1. Concentration of ytterbium (mg.kg^{-1}) ± S.D. in feed samples pelleted three times, dashed line indicates the formulated concentration of Yb in the feed. (F = 1.016, df = 2, 15, P = 0.386). $P < 0.05$ was considered significant.

2.5.2 Pellet water stability

The feed pellets of each treatment remained visually intact over the two feeding periods (9 and 15 h) during the experiment. The majority of the feed was consumed after the first hour of introducing feed to prawns, therefore dry matter (DM) loss after 1 h was used to determine pellet water stability. FI was adjusted to account for the DM loss during this period. The commercial reference feed treatment (Ridley Prawn Starter Enhance) had a significantly lower pellet stability (97.32 ± 0.53% DM) compared to the basal, F50 and FV90 feed treatments; 99.18 ± 0.09, 99.21 ± 0.28 and 99.61 ± 0.08 % DM respectively (P = 0.004), although similar to UP85 and MMB feed treatments (Table 2.3).

2.5.3 Growth performance

Initial mean starting weights (2.025 ± 0.029 g) of *P. monodon* were not significantly different amongst all treatments (P = 0.534) on day 1 of the experiment (Table 2.4). After 15 d the mean final weights (4.004 ± 0.070 g) and weight gains (1.979 ± 0.068 g) of prawns were not different between treatments (P = 0.828 and 0.857 respectively) (Table 2.4). Mean daily feed intake for prawns fed all feed treatments were not significantly different and ranged from 0.98 ± 0.07 g DM Pd^{-1} for prawns fed the commercial feed up to 1.23 ± 0.02 g DM Pd^{-1} when fed the basal feed. Specific growth rates (SGR) ranged from 4.34 ± 0.26% d^{-1} for prawns fed the basal treatment up to 4.79 ± 0.31% d^{-1} when fed the marine macro-algae blend meal treatment, with no significant difference between treatments (P = 0.885). Similarly, when growth was expressed as average weekly gain (AWG) weight gain ranged from 0.87 ± 0.07 g wk^{-1} for prawns fed the basal treatment up to 0.99 ± 0.08 g wk^{-1} when fed marine macro-algae blend meal (MMB), and was not significantly different (P = 0.857) (Table 2.4). The FER of prawns across all treatments were not significantly different after feeding for 15 d, prawns fed the basal feed had the lowest FER at 59.98 ± 4.10% and prawns

fed the Ridley feed had the highest FER of 75.67 ± 5.44% (Table 2.4). The survival rates of prawns were not significantly different between treatments and ranged from 70.83 ± 7.97 up to 95.83 ± 4.16% (Table 2.4).

2.5.4 Immune response

There were no significant differences in total haemocyte counts (THC) of prawns fed all treatments ($P = 0.475$), prawns fed the basal feed had a THC of 3.42 ± 0.31 cells x 10^7 ml^{-1} and prawns fed the Ridley feed had a THC of 4.02 ± 0.26 cells x 10^7 ml^{-1} (Table 2.5). When haemocytes were expressed as a percentage of non-granular (hyaline) and granulocytes (DHC) there were no significant differences between treatments ($P = 0.196$). However, when haemocytes were quantified by granulocytes per.ml^{-1} (GHC), prawns fed the FV90 feed treatment had a significantly higher ($P = 0.036$) number of granulocytes ml^{-1} (1.72 ± 0.25 cells x 10^7 ml^{-1}) when compared to prawns fed F50 (1.02 ± 0.11 cells x 10^7 ml^{-1}), but were not different to prawns fed all other feeds (Figure 2.2). Prawns fed the basal feed had significantly higher ($P = 0.001$) PO activity (0.028 ± <0.01Δ Abs/min^{-1}) than prawns fed the Ridley, UP85 and F50 feeds, however PO activity was not different when compared to prawns fed the FV90 and MMB feeds (Figure 2.3).

2.5.5 Digestive gland histopathology

Transverse sections of digestive gland tissue in all prawns from all feed treatments presented as tightly packed tubules contained within the inter-connective tissue of the mid-region of digestive gland tubules (Figure 2.4). Digestive glands were of star-shaped appearance, indicative of a healthy fasted animal while granular material present within the central lumen (Lu) consists of secreted material from cellular mechanisms (Figure 2.4). Cells contained

within each tubule epithelium consists of predominantly B- (blister-like), F- (fibrillar) and R-cells (resorptive) (Figure 2.4A to 2.4F). Also noted are examples of lipid deposits (Ld) within the R-cells, ranging from small to large lipid vacuoles.

Table 2.3. Pellet stability (mean ± S.E., n = 3) of experimental and reference feeds after submersion in water for 1 h (df = 5, 12).

	Units	**Feeds**						**One-way ANOVA**	
		BASAL	**RIDLEY**	**FV90**	**UP85**	**F50**	**MMB**	**F-value**	***P***
1 h	% DM	99.18 ± 0.09[a]	97.32 ± 0.53[b]	99.61 ± 0.08[a]	98.24 ± 0.09[ab]	99.21 ± 0.28[a]	98.72 ± 0.51[ab]	6.249	0.004

Feeds contain: BASAL = Control, RIDLEY = Prawn Starter Enhance, UP85 = Maritech® *U. pinnatifida*, FV90 = Maritech® *F. vesiculosus*, F50 = Fucorich® *U. pinnatifida* extract, MMB = marine macro-algae blend meal (50:50 *U. pinnatifida* and *A. nodosum*).
Means with similar superscripts are not significantly different ($P < 0.05$ was considered significant, n = 3).

Table 2.4. Growth performance and feed efficiencies (mean ± S.E., n = 4) of juvenile *Penaeus monodon* fed feeds containing different sources and inclusions of fucoidan over 15 d (df = 5, 18).

	Units	**Feeds**						**One-way ANOVA**	
		BASAL	**RIDLEY**	**FV90**	**UP85**	**F50**	**MMB**	**F-value**	***P***
Initial weight	g	2.023 ± 0.018	2.013 ± 0.006	2.011 ± 0.022	2.026 ± 0.006	2.051 ± 0.015	2.025 ± 0.018	0.847	0.534
Final weight	g	3.894 ± 0.181	3.982 ± 0.088	3.928 ± 0.249	3.914 ± 0.194	4.142 ± 0.186	4.163 ± 0.162	0.422	0.828
Feed intake[1]	g DM Pd^{-1}	1.23 ± 0.02	0.98 ± 0.07	1.12 ± 0.05	1.06 ± 0.05	1.17 ± 0.05	1.04 ± 0.09	2.140	0.107
Weight gain[2]	g	1.871 ± 0.164	1.969 ± 0.087	1.917 ± 0.235	1.887 ± 0.196	2.091 ± 0.189	2.138 ± 0.179	0.378	0.857
Specific growth rate[3]	% d^{-1}	4.34 ± 0.26	4.54 ± 0.14	4.42 ± 0.40	4.36 ± 0.33	4.66 ± 0.32	4.79 ± 0.31	0.334	0.885
Average weekly gain[4]	g wk^{-1}	0.87 ± 0.07	0.91 ± 0.04	0.89 ± 0.11	0.88 ± 0.09	0.97 ± 0.08	0.99 ± 0.08	0.378	0.857
Feed efficiency ratio[5]	%	59.98 ± 4.10	75.67 ± 5.44	63.79 ± 7.18	67.04 ± 7.22	66.62 ± 5.41	74.20 ± 6.90	0.962	0.467
Survival	%	95.83 ± 4.16	87.50 ± 7.97	79.16 ± 4.16	83.33 ± 11.78	75.00 ± 4.81	70.83 ± 7.97	1.500	0.239

Feeds contain: BASAL = Control, RIDLEY = Prawn Starter Enhance, FV90 = Maritech® *F. vesiculosus*, UP85 = Maritech® *U. pinnatifida*, F50 = Fucorich® *U. pinnatifida* extract, MMB = marine macro-algae blend meal (50:50 *U. pinnatifida* and *A. nodosum*).

[1]Feed intake (g DM Pd^{-1}) = (dry matter of feed fed, after adjustments for uneaten feed and leaching) / mean prawn days (Pd^{-1})).

[2]Weight gain (g) = final wet weight – initial wet weight.

[3]Specific growth rate (% d^{-1}) = ln(final wt) – ln(initial wt) x 100 / days of experiment.

[4]Average weekly gain (g wk^{-1}) = (wet weight final – wet weight initial / weeks of experiment.

[5]Feed efficiency ratio (%) = (mean wet weight gain (g) / feed intake (g DM mean Pd^{-1}) after adjustments for uneaten feed and leaching) x 100.

$P < 0.05$ was considered significant.

Table 2.5. Innate immune responses (mean ± S.E., n = 4) of juvenile *Penaeus monodon* fed feeds containing different sources and inclusions of fucoidan over 15 d (df = 5, 18).

		Feeds							One-way ANOVA	
	Units	**INITIAL**	**BASAL**	**RIDLEY**	**FV90**	**UP85**	**F50**	**MMB**	**F-value**	***P***
THC	cells.ml^{-1} x 10^7	1.44 ± 0.39	3.42 ± 0.31	4.16 ± 0.64	4.73 ± 0.76	3.66 ± 0.34	3.58 ± 0.48	4.12 ± 0.16	0.948	0.475
DHC	%	29.88 ± 3.44	32.16 ± 3.41	29.54 ± 1.72	36.83 ± 2.49	32.08 ± 2.08	28.91 ± 1.28	34.20 ± 2.12	1.658	0.196
GHC	cells.ml^{-1} x 10^7	0.51 ± 0.18	1.20 ± 0.12^{ab}	1.07 ± 0.08^{ab}	1.72 ± 0.25^{a}	1.18 ± 0.14^{ab}	1.02 ± 0.11^{b}	1.41 ± 0.11^{ab}	3.051	0.036
PO activity	Δ Abs/min^{-1}	0.022 ± <0.01	0.028 ± <0.01^{a}	0.012 ± <0.01^{b}	0.016 ± <0.01^{ab}	0.009 ± <0.01^{b}	0.013 ± <0.01^{b}	0.018 ± <0.01^{ab}	6.726	0.001

Feeds contain: BASAL = Control, RIDLEY = Prawn Starter Enhance, UP85 = Maritech® *U. pinnatifida*, FV90 = Maritech® *F. vesiculosus*, F50 = Fucorich® *U. pinnatifida* extract, MMB = marine macro-algae blend meal (50:50 *U. pinnatifida* and *A. nodosum*).
Means with different superscripts are significantly different ($P < 0.05$ was considered significant). Initial baseline group are not included in statistics.

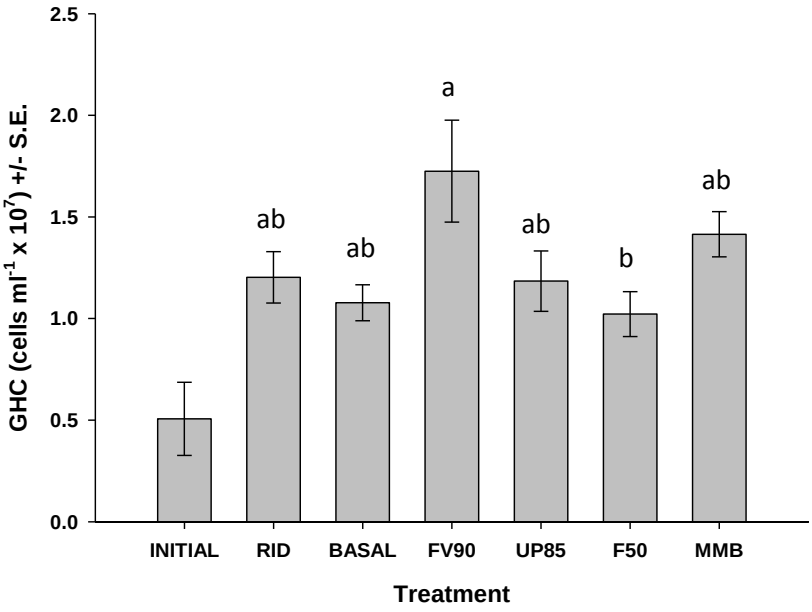

Figure 2.2. Mean number of *Penaeus monodon* granular haemocytes (GHC) per.ml^{-1} of haemolymph ± S.E. Means with different superscripts are significantly different (P = <0.05, n = 4), Initial baseline group are not included in statistics (F = 3.051, df = 5, 18, P = 0.036). P < 0.05 was considered significant.

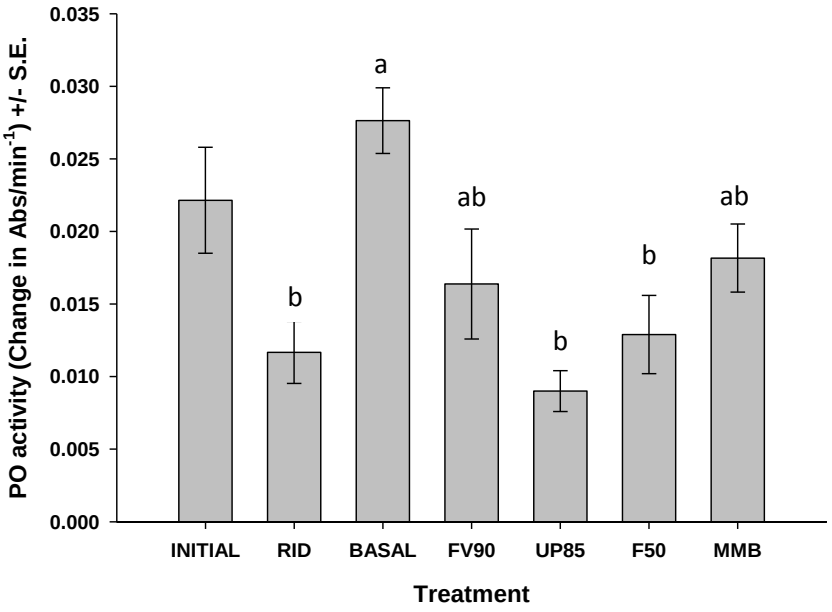

Figure 2.3. Mean phenoloxidase activity (PO activity) of *Penaeus monodon* expressed as change in Abs/min/100 µl haemolymph ± S.E. Means with different superscripts are significantly different (P = <0.05, n = 4), Initial group are not included in statistics (F = 6.726, df =5, 18, P = 0.001). $P < 0.05$ was considered significant.

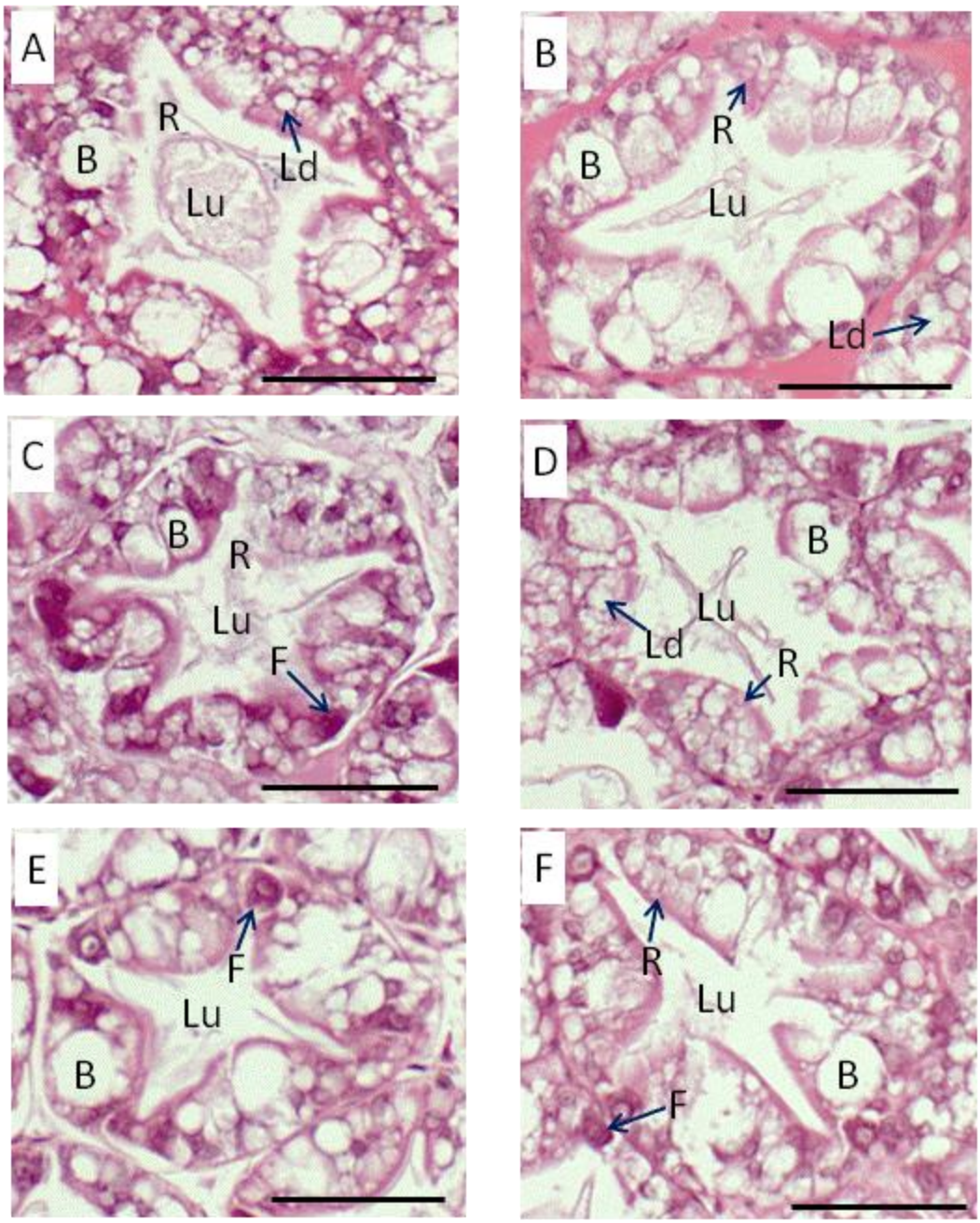

Figure 2.4. Transverse sections of digestive gland tubules of juvenile *Penaeus monodon* fed; basal feed (A), Ridley (B), FV90 (C), UP85 (D), F50 (E) and MMB (F) for 15 d. Sections were stained with haematoxylin and eosin, and show tubule lumen (Lu), B-cells (B), F-cells and lipid droplets (Ld) within R-cells, scale bar = 100 µm. Magnification = 200 x light microscopy.

2.6 Discussion

The use of fucoidan in prawn feeds has many positive benefits such as enhancing host innate immune responses, disease resistance to viral and bacterial pathogens and increased growth performance. In the current study, four commercially available fucoidan products were tested in *Penaeus monodon* feeds and were compared to a control and a commercial reference feed. There were limited differences in the measured responses between fucoidan fed prawns when compared to prawns fed the basal feed by the conclusion of the 15 d feeding trial. Prawns fed FV90 had on average 59.3% more granular haemocytes per ml than prawns fed F50; however prawns fed other treatments were not different. Granular haemocytes (small and large) contain the prophenoloxidase system (proPO) and upon contact with polysaccharides, β-1,3-glucans or lipopolysaccharides the granular haemocytes de-granulate activating the immune system (Johansson and Soderhall, 1985; Takahashi et al., 2000; Vargas-Albores and Plascencia, 2000; Felix et al., 2004; Kitikiew et al., 2013). However, prawns fed the FV90 and F50 feeds had similar mean PO activity values and were lower than the PO activity of prawns fed the basal feed, containing no fucoidan. Within the literature the PO activity of unchallenged controls are always lower than the immunostimulant treatment, this is due to the immune system of the control group not being stimulated to act upon foreign substances, such as immunostimulants.

The THCs were similar for all prawns no matter if they were fed a fucoidan or non-fucoidan feed. The range of total haemocytes per ml in the current study were similar to Traifalgar et al. (2010) who fed *U. pinnatifida* to *Marsupenaeus japonicus* at 500 and 1000 $mg.kg^{-1}$ for 8 weeks. In contrast *Litopenaeus vannamei* fed Ergosan (unspecified algal product containing 1% alginic acid from *Laminaria digitata*) for 15 d observed no difference in THC when compared to the control, although there was a significantly higher number of granular cells in prawns fed the control (Montero-Rocha et al., 2006). When fucoidan was included in *L.*

vannamei feeds at 500, 1000 and 2000 g.kg^{-1} there was an increase in the THC and PO activity of prawns at 14 d this increased further at 21 d (Kitikiew et al., 2013). The lack of stimulation in measured THC, GHC and PO activity in prawns fed fucoidan in the current study when compared to the control may be explained by a lower inclusion of fucoidan in the feeds, which may be too low to cause an effect of stimulation. Also the duration of the feeding trial may be too short to determine changes in immune responses, in particular when a lower fucoidan inclusion rate is used in *P. monodon* feeds. Prawns fed feeds containing fucoidan had similar immune responses to the prawns fed the commercial feed (Ridley Aqua-Feeds); therefore prawns fed fucoidan performed the same as the commercial standard. Commercial prawn feeds manufacturers do not disclose the ingredient composition of their feeds, which may or may not contain an immune stimulatory product.

It is plausible that the measured immune responses (THC, DHC and PO activity) could still be increasing after 15 d. Therefore, the current trial may not have captured the potential of the tested ingredients in this study. Other factors for the limited variation in immune responses may have been due to the nature of the culture environment, which was conducted under optimum culture conditions and without an elicitor or challenge within the study, therefore the prawns may have not elicited an immune response as stress may have not been a factor in the current experiment.

Growth performance of *P. monodon* was not enhanced by any particular feed treatment after 15 d. Prawns fed experimental feeds were comparable to prawns fed the commercial reference feed; therefore fucoidan has not caused reduced growth rates. On average prawns grew at 4.52% d^{-1} (SGR), and average weight gain was 1.98 g. When fucoidan was fed to *Marsupenaeus japonicus* for 8 weeks the average SGR ranged from 1.8% d^{-1} (100 mg.kg^{-1} fucoidan) to1.9 and 2.06% d^{-1} (500 and 1000 mg.kg^{-1}) respectively in comparison to the control (1.6% d^{-1}) treatment (Traifalgar et al., 2010). In the current experiment SGR was

much greater than in the previous study (Traifalgar et al. 2010) which used prawns of similar size. The rapid growth indicates experimental feeds did not hinder growth performance.

Survival of *P. monodon* was not influenced by the inclusion of fucoidan in feeds (81.94 ± 3.67%). Mortality during the trial was always associated with a moulted animal, occurring predominately during the dark hours. Prawns that died while soft-bodied from predation were removed as they were noticed, reducing compounding factors associated with prawns consuming feed sources other than experimental feeds. It has been demonstrated that the mean survival of *M. japonicus* fed feeds containing fucoidan after 8 weeks ranged between 80 - 86.7% (Traifalgar et al., 2010). In the current study the mean survival ranged between 70.83 ± 7.97 (MMB) up to 95.83 ± 4.16 (basal) after only 15 d. Prawns were fed twice daily and additional hides were placed within tanks to help increase survival, therefore feeding prawns more regularly may increase survival over an extended trial duration.

Immunostimulants incorporated into aqua-feeds are included at very low proportions when compared to the proportions of other ingredients within the feed. The concentration of Yb in the dough was not affected by the number of times the dough was pelleted; therefore the feeds were pelleted twice, as normal operating temperatures were reached by the second pelleting time.

Pellet water stability over a one hour period was high across all experimental treatments, while the commercial reference feed had the lowest stability (97.32%). This is due to the different type of functional ingredients within the commercial feed formulation. The commercial feed was supplied as a mash and pelleted using the same equipment as the experimental feeds. In the current experiment all pellet water stabilities were higher than that claimed by Cruz-Suarez et al. (2009) who included seaweed meals at 33 $g.kg^{-1}$ suggesting functional properties of other ingredients may have contributed to the high water stability.

The appearance of digestive gland structures of all prawns fed experimental feeds was comparable to healthy prawns as described by Bell and Lightner (1988). Changes within the digestive gland structure have been shown to occur relatively quickly; for instance, Piedad-Pascual et al. (1983) observed changes including complete loss of tubule organisation, leaving only thin connective tissue capsules after feeding 10% molasses for 14 d. Pathology of digestive glands, including sloughed cells, enlarged B-cell vacuoles and loss of acinar structure has been associated with feeding cow pea and mung beans to *P. monodon* for 20 d (Kumaraguru vasagam et al., 2007). Therefore, if there were ANFs or toxic substances in the experimental feeds, pathological changes should have presented within the duration of the current study.

2.7 Conclusion

This screening trial demonstrated that test ingredients were all potentially useful, no toxic effects were determined and prawns consumed feeds resulting in high growth rates. Future studies using the current fucoidan products need to consider a graded series of inclusion rates or an inclusion rate within the literature which has elicited an immune response. The feeding trial duration prior to testing immune responses should be longer to determine peak immune status. Further investigation of the current experimental feeds need to be determined after challenging prawns with a stressor, such as a combined drop in water temperature and salinity, as experienced in commercial prawn culture after the onset of the monsoonal season that is associated with acute expression of GAV.

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Growth performance of *Penaeus monodon* after feeding on feeds containing fucoidan, and the effect of an acute temperature and salinity drop on immune response

CHAPTER III

3.1. Abstract

Sub-adult *Penaeus monodon* were fed for 21 days on three experimental feeds containing fucoidan at a 1g.kg^{-1} inclusion level, and were compared to a control feed for performance. Prawns were fed four times daily to satiation and reared at 30°C water temperature and 40‰ salinity. At the conclusion of the feeding trial intermoult prawns were moved to another system where they were either held at the same water temperature and salinity (control) or at a reduced temperature and salinity (24°C and 20‰, stress treatment). Feed intake (FI), specific growth rate (SGR), average weekly gain (AWG), survival and final prawn weights were not significantly different between all treatments tested. Feeds had no significant effect on the whole body chemical composition (crude protein, crude lipid, gross energy and moisture). A combined drop in water temperature and salinity had a significant impact on the number of circulating haemocytes. Mean total haemocyte numbers (THC) decreased on average by 18.59% for prawns stressed by decreasing water temperature and salinity. Similarly granular haemocyte counts (GHC) on average decreased by 17.62%. However, there was no effect of feed treatment or stress on PO activity.

3.2 Introduction

Daily and seasonal fluctuations in environmental conditions can cause stress to farmed prawns including *Penaeus monodon*, as they are contained within a defined body of water (Le Moullac and Haffner, 2000). The stress tolerance of a prawn is dependent upon the moult stage, nutritional and disease status of the prawns (Lignot et al., 1999; Lignot et al., 2000; Le Moullac and Haffner, 2000; Liu et al., 2004; de la Vega et al., 2006). In the culture environment, water temperature and salinity influence the general homeostasis of prawns, affecting metabolism, growth, immune response and disease resistance (Staples and Heales, 1991; Jobling, 1994; Le Moullac and Haffner, 2000; Lin et al., 2012). During commercial prawn production, the water temperature can reach 30°C and salinity 40‰. However during periods of high rainfall such as the start of the monsoonal season, both water temperature and salinity can drop to 22°C and 10‰ within 2 to 3 days as a result of cooler rainwater mixing with the warmer seawater (Matt West, Pers. Comm.).

The metabolic rate of an aquatic animal is temperature dependent, where the metabolic pathways increase with increasing body temperature, within thermal tolerance limits for a specific species (Jobling, 1994). *P. monodon* is a euryhaline species capable of hypo- and hyper-osmotic regulation in a wide range of salinities (3-50‰) (Rajesh et al., 2012). Fluctuations in salinity affect cellular processes such as osmoregulation; impaired osmoregulation can impact the overall gross metabolic rates of prawns, a requirement to maintain osmotic homeostasis, typically expressed as oxygen consumption (Mantel and Farmer, 1983; Pequeux, 1995). Growth rates in prawns have been shown to be affected by water temperature. *Penaeus merguiensis* increased their growth rates when cultured at 25°C when compared to culture temperatures of 20°C and

lower (Staples and Heales 1991). This was further supported by increased growth rates of *P. merguiensis* cultured at 24°C when compared to 21°C (Hoang et al., 2002).

Haemocytes (hyaline cells, small granular and large granular cells) are responsible for sealing off wounds, phagocytosis, encapsulation, coagulation and melanisation from the prophenoloxidase (proPO) pathway (Smith and Soderhall, 1983; Johansson et al., 2000; Smith et al., 2003). Changes in haemocyte mediated immune parameters including total haemocyte count (THC), granular haemocyte counts (DHC) and phenoloxidase activity (PO) are commonly used to assess stress effects as haemocyte numbers also change in relation to acute water temperature and salinity changes (Cheng et al., 2005; Lin et al., 2012). A decrease in salinity from 27 to 20‰ prior to a *Vibrio harveyi* disease challenge resulted in increased disease-related mortality in *P. monodon* (Prayitno and Latchford, 1995), demonstrating that prawns cultured at lower salinities are more vulnerable to pathogens. Gill-Associated Virus (GAV) causes a disruption in homeostasis and the resulting stress can initiate an acute viral infection resulting in a significant loss of prawns (de la Vega et al., 2004; Liu et al., 2006).

The delivery of antiviral medication or prophylactic chemicals via the feed is well documented in vertebrates (Heinzelmann et al., 1998; Berteau and Mulloy, 2003; Hayashi et al., 2008; Azuma et al., 2012), and feeding experiments in other penaeids have shown some promise.

Fucoidan, a sulphated polysaccharide from brown macro-algae (*Phaeophyceae*) contains L-fucose. It has numerous biological properties resulting in: enhanced growth in *Marsupenaeus japonicus* (Traifalgar et al., 2010), increased immune responses in *M. japonicus* (Traifalgar et al., 2010) and *P. monodon* (Immanuel et al., 2012), and increased disease resistance in *P. monodon* (Chotigeat et al., 2004; Immanuel et al., 2012) and *M. japonicus* (Takahashi et al., 1998).

There has been a considerable amount of research investigating how environmental stress impacts the innate immune system in invertebrates; however there is limited research assessing the effects of immunostimulants in response to environmental stressors.

The aims of this study were:

- To determine whether feeding commercially available fucoidan for 21 days enhances growth performance and whole body chemical composition of *P. monodon*.
- To measure haemocyte mediated immune responses (THC, DHC and PO) of *P. monodon* after a combined decrease in water temperature and salinity, in comparison to non-stress control groups fed feeds with and without fucoidan.

3.3 Materials and methods

3.3.1 Prawn stock

Fourteen day old black tiger prawn (*P. monodon*) postlarvae (PL14) were obtained from Rocky Point Prawn Farm, Gold Coast, Queensland, Australia. Postlarvae were transported by air to the University of Tasmania, Launceston, Tasmania where they were held under quarantine for the duration of the experiment (Special Authority Number, SA 12-21). Approximately 10,000 PL were on-grown to approximately 0.5 g, in two raceway tanks within a recirculating seawater system. Postlarvae were fed a mixture of live *Artemia* spp. and commercial prawn crumble (Prawn Starter Enhance MR, Ridley Aqua-Feed) three times daily to satiation until PL 28. Thereafter, PL were fed to satiation twice daily on a commercial prawn feed at 0800 and 1700 hrs by hand, with an additional 4 feeds (2000, 2300, 0300 and 0500 hrs) from 2 digital automatic feeders (Automatic Fish Feeder, FF3), located on top of each raceway.

Postlarvae were maintained under optimum environmental conditions (salinity: 34-37‰; photoperiod: 12:12 light dark cycle; temperature: 28-30°C) and water quality parameters (NH_3: < 0.5 ppm, NO_2: < 0.5 ppm, NO_3: < 20 ppm, pH: 8.2, using a saltwater master test kit (API™).

3.3.2 Experimental system

The recirculating seawater system comprised of twenty four 118 L rectangular (surface area = 0.43 m²). Food grade white high density polypropylene tanks were used. Water quality was monitored daily (NH_3: <0.25 ppm, NO_2: <1.0 ppm, NO_3: <20 ppm, pH: 8.1, KH: 161.1 ppm and Ca^{2+}: 450 ppm) using saltwater master test, calcium and carbonate hardness kits (API™) and maintained by biofiltration, UV sterilization (18 W Emperor Aquatic's) and solids removal. Average water temperature was 30.75 ± 0.01°C and was heated by an 8.1 kW reverse cycle air

conditioner (Mitsubishi Electric, MUZ-GA71VA) and a submerged water heater (Aqua One, 60 W), providing additional heating. Average salinity was 39.29 ± 0.22‰ and was measured daily using a salinity refractometer (IWAKI, TD5Q8). Salinity was adjusted as required by the addition of de-chlorinated tap water or pool salt (Cheetham Salt, Mermaid Finest, Australia) while continuous aeration was provided by individual air-stones in each tank. Tanks were fitted with grey PVC hinged tank covers to stop prawns escaping, and tank outlets were screened with fibreglass flyscreens (1 mm^2) to keep prawns within tanks. The average flow rate to tanks was 2.3 $L.min^{-1}$; exchanging each tank volume every 51 mins.

3.3.3 Feed formulation

The basal feed was formulated at 47% crude protein, 7.3% crude lipid and 16.2 $MJ.kg^{-1}$ of energy 'as is' (Table 3.1). The basal feed (control) was formulated to contain 2.0 $g.kg^{-1}$ α-cellulose (Sigma-Aldrich), and was partially or completely substituted with the fucoidan test ingredients to provide a fucoidan concentration of 1000 $mg.kg^{-1}$. Fucoidan test ingredients were: Maritech® *Fucus vesiculosus* extract 92.6% fucoidan (FV90), Maritech® *Undaria pinnatifida* extract 89.0% fucoidan (UP85) and Fucorich® *Undaria pinnatifida* extract 50.7% fucoidan [(F50) Marinova Pty Ltd, Cambridge, Tasmania, Australia].

Fish meal (Skretting Australia, Cambridge, Tasmania) and South American krill meal (Ridley Aqua-Feeds, Narangba, Queensland) were included as sources of protein and lipid, while krill meal was also a feed attractant. Wheat flour (Four Roses, New South Wales, Australia) and wheat gluten (MP Biomedicals) were binders. Cholesterol (Sigma-Aldrich) was added at 1 $g.kg^{-1}$ and soy lecithin (Lucas Meyer Eurologistic, IIIinois) at 10 $g.kg^{-1}$. Macro minerals premix was

made from individual minerals (Sigma-Aldrich) and were included at 33 $g.kg^{-1}$, containing the recommended mineral concentrations for marine shrimp (Davis and Lawrence, 1997). Vitamin and micro mineral premix was made from individual vitamins and minerals (Sigma-Aldrich) and was added at 1 $g.kg^{-1}$ according to the recommended vitamin and mineral concentrations for marine shrimp (Conklin, 1997; Davis and Lawrence, 1997) (Table 3.1).

Fish meal was sifted through a 1 mm sieve (Greer and Ashburner, 1-C). Dry bulk ingredients were homogenised in a mixer (Brice Australia, Pty Ltd, VFM-20C) for 20 min before small dry ingredients including test ingredients were homogenised in a subsample of the bulk mix, then returned to the bulk mix and mixed for further 20 min. Lecithin and choline chloride [(MP Biomedicals) dissolved in distilled water] and distilled water were added to the dry mixture and further mixed for 20 mins until a crumbly dough could be formed. The dough was then passed twice through a 2 mm die of a commercial pasta maker (ItalPast, Mac 10s) and cut to approximately 6 mm lengths. Pellets were steamed for 10 min (Tefal, Steam Cuisine) before being air dried (Forma Scientific, 68732-1) until feeds contained less than 10% moisture. To determine moisture content of feeds, sub-samples of 2 g were dried for 2 h at 135°C. Feeds were stored in airtight bags at -20°C until required.

Table 3.1. Ingredient inclusion as is ($g.kg^{-1}$) and mean chemical composition (n=2) of experimental feeds ($g.kg^{-1}$ DM) ± S.E.

	Feeds			
Ingredients (as is)	**BASAL**	**FV90**	**UP85**	**F50**
Fishmeal	500.00	500.00	500.00	500.00
Wheat flour	320.70	320.70	320.70	320.70
Krill meal	60.00	60.00	60.00	60.00
Wheat gluten	70.00	70.00	70.00	70.00
Test ingredient[1]	0.00	1.08	1.13	1.97
α-cellulose	2.00	0.92	0.87	0.03
Macro mineral mix[2]	33.00	33.00	33.00	33.00
Lecithin	10.00	10.00	10.00	10.00
Vitamins & micro mineral mix[3]	1.00	1.00	1.00	1.00
Cholesterol	1.00	1.00	1.00	1.00
Stay C	1.00	1.00	1.00	1.00
Pigment	0.50	0.50	0.50	0.50
Banox E	0.20	0.20	0.20	0.20
Choline chloride	0.60	0.60	0.60	0.60
Chemical composition $g.kg^{-1}$				
Dry matter	911.19 ± 1.55	903.05 ± 0.98	932.87 ± 1.15	928.12 ± 0.39
Crude protein	551.41 ± 1.01	542.79 ± 3.81	540.32 ± 10.05	526.86 ± 2.31
Crude lipid	96.83 ± 1.89	99.02 ± 0.90	96.02 ± 1.74	99.54 ± 0.55
Gross energy ($MJ.kg^{-1}$)	18.84 ± 0.05	18.78 ± 0.04	19.18 ± 0.01	18.80 ± 0.06
Ash	123.42 ± 0.82	124.39 ± 0.21	124.37 ± 0.35	127.41 ± 0.40
Protein:energy (g CP.MJ GE^{-1})	29.42	28.90	28.17	28.02

Feeds contain: BASAL = Control, FV90 = Maritech® *Fucus vesiculosus*, UP85 = Maritech® *Undaria pinnatifida*, F50 = Fucorich® *Undaria pinnatifida* extract.
[1]Fucoidan ingredients (Marinova Pty Ltd).
[2]Macro mineral mix, (g.kg): K_2PO_4, 10; $Ca(H_2PO_4)_2$, 10; NaH2PO4, 10; $MgSO_4.7H_2O$, 0.3 (Davis and Lawrence, 1997).
[3]Vitamin and micro mineral premix. Vitamins (mg.kg): thiamine-HCl, 60; riboflavin, 25; nicotinic acid, 40; pyridoxine-HCl, 50; pantothenic acid, 75; biotin, 1; folic acid, 10; vitamin B12, 0.2; myo-inositol, 400; L-ascorbic acid, 200; vitamin A acetate, 10; DL-α-tocopherol acetate, 100; vitamin D3, 0.1; menadone sodium bisulfite, 5, α-cellulose, 23.7. Micro minerals (mg.kg): $CuSO_4.5H_2O$, 32; $ZnSO_4.7H_2O$, 15; Na_2SeO_3, 0.2; α-cellulose, 52 (Conklin, 1997; Davis and Lawrence, 1997).

3.3.4 Pellet stability

The majority of feed was consumed by the prawns after 1 h; therefore pellet stability was determined for each feed treatment after 1 h, to adjust feed intake data for dry matter (DM) loss due to leaching during this period. Pellet stability (1 h) was conducted by immersing triplicate samples (~ 2 g) of each feed treatment in the same manner as per normal feeding, accounting for water temperature, salinity, aeration and flow rates, however without prawns. Pellets of a known weight were spread around the tanks and after 1 h pellets were siphoned using a vinyl hose of 8 mm in diameter onto a 263 µm mesh screen. Pellets were then transferred to an aluminium foil pan, dried in an oven at 135°C for 4 h and, allowed to cool to room temperature in a desiccator before weighing. Pellet stability was calculated as the final DM expressed as a percentage of the initial DM in pellets.

3.4 Experimental period

3.4.1 Acclimation

Eight *P. monodon* (mean weight 5.16 ± 0.06 g) were stocked randomly into each tank of the experimental system. Prawns were stocked to four tanks per day to achieve a staggered start; to ensure that the prawns were fed feeds for the same duration at the time of final sampling. Prawns were fed a basal feed (control) four times per day (0730, 1200, 1600 and 1930 hrs) to satiation for 7 d of diet acclimation; prior to the 0730 hr feed, all uneaten pellets, faeces and exuvia were siphoned from tanks. During the 7 day acclimation period dead animals were replaced with animals from the same cohort in additional tanks within the same system. Two artificial hides made from bunched strips of fibreglass flyscreen mesh material suspended below a block of polystyrene were placed in each tank to increase survival of moulting animals while soft-bodied.

3.4.2 Experiment

At the start of the experiment all prawns were blotted dry with paper towel and individually weighed by placing prawns in a tared closed container on a balance (AND, FX300). Seven prawns (mean weight 6.27 ± 0.19 g) were returned to the tanks to reduce stocking density due to the increased size of the prawns. Feed treatments were assigned to tanks in blocks of four tanks and fed experimental feeds as per section 3.4.1. At this time 12 tanks were predetermined to be either "stress" or "non-stress" treatments for final sampling at the conclusion of the feeding trial. The experiment was concluded 21 d later. Prawns were fasted for 24 h before being individually weighed and moult staged (Smith and Dall, 1985). Three intermoult (stage C) prawns per tank were placed in one prawn trap made of a fine mesh material. Prawns from each of the four tank block were submerged in one 118 L polypropylene tank for 3 h at either 30°C and 40‰ salinity (non-stress group) or 24°C and 20‰ salinity (stress group), with constant aeration.

After 3 h prawns were weighed and moult staged to ensure that they were in intermoult stage. Haemolymph was removed from the cardiac sinus using a 25 G needle and 1 ml tuberculin syringe, rinsed with chilled Shrimp Salt Solution (SSS) (450 mM NaCl, 10 mM KCl, 10 mM EDTA.Na_2, 10 mM HEPES, pH 7.3, 850 mOsm kg^{-1}) (Vargas-Albores et al., 1993). Haemolymph was immediately ejected into a 2 ml microcentrifuge tube. Two aliquots of the haemolymph were quickly removed and added to individual tubes: 50 µl haemolymph in 200 µl SSS for phenoloxidase activity (PO activity) and 25 µl haemolymph in 25 µl of formalin containing 10% NaCl for total haemocyte counts (THC) and differential haemocyte counts (DHC). At the conclusion of the feeding trial, 3 prawns per tank in intermoult were taken for whole chemical composition and snap-frozen in liquid nitrogen prior to storing at -20°C.

Initial baseline measures from 12 prawns were taken for THC, DHC, PO activity and viral loading as previously described for final sampling, and twelve prawns from the same stock culture were sampled for whole body chemical composition.

3.5 Immune response parameters

3.5.1 Differential haemocyte count (DHC)

Formalin fixed haemolymph was used for DHC according to Sritunyalucksana et al. (2005). After fixing haemocytes in formalin for approximately 20 min, 50 µl of Rose Bengal solution (Sigma-Aldrich, 1.2% Rose Bengal in 50% ethanol) was added to the micro-centrifuge tube and incubated at room temperature for 20 min (Sritunyalucksana et al., 2005). Stained haemolymph smears from individual prawns were made using one drop (20 µl) of stained haemolymph solution smeared onto a clean microscope slide. Smears were air-dried, then counterstained with haematoxylin (Sigma-Aldrich) for 3 min, before rinsing in tap water for 2 min followed by immersion for 1 min in 95% iso-propyl alcohol and 2 min in 100% iso-propyl alcohol (repeated twice), using an automated diversified stainer (Sakura, DRS-60) (Sritunyalucksana et al., 2005). Following dehydration, slides were placed in xylene for 2 min and mounted with a cover-slip using DPX mountant (Sigma-Aldrich). Granulocyte ratios (DHC) were determined by counting 200 haemocytes per smear under 400 x objective of a light microscope (Olympus BH-2) and expressed as a proportion of granulocytes (GHC) (small-granular and large-granular haemocytes) in 200 total haemocytes, i.e. count/200 x THC (Sritunyalucksana et al., 2005).

3.5.2 Total haemocyte count (THC)

One drop of Rose Bengal stained pooled haemolymph mixture from DHC samples was placed on a Neubauer Improved Bright-line haemocytometer under 400 x magnification of a light microscope (Olympus, CH30). At least 200 haemocytes were counted in triplicate for quantifying the total number of haemocytes per ml^{-1} of haemolymph.

3.5.3 Phenoloxidase (PO) activity

The PO activity method followed the protocol from Hernandez-Lopez et al. (1996) using the modified 96 well micro-plate method. Haemolymph diluted in SSS (1 in 4 dilution) was centrifuged (Eppendorf, S415D) at 300 x *g* at 4°C for 5 min and the supernatant was removed. The cell pellet was resuspended with 200 µl cacodylate buffer (10 mM sodium cacodylate, 10 mM $CaCl_2$, pH 7.0).

PO activity was measured spectrophotometrically (in triplicate) by the formation of dopachrome from the substrate, L-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich). Fifty microliters of pooled haemocyte cell suspension was incubated with 50 µl of laminarin (Sigma-Aldrich) (1 mg/ml in cacodylate buffer) in a flat-bottomed 96 well micro-plate (TTP®, 92696) for 30 min at 25°C. Fifty microliters of L-DOPA (3 mg/ml in cacodylate buffer) was added to each well and incubated at 25°C for 10 min, before reading the optical density at 492 nm on an ELISA reader (SPECTRA, Rainbow Thermo). Enzyme activity was expressed as the change in absorbance per min (Δ $Abs.min^{-1}$) per 100 µl of haemolymph.

3.6 Chemical analyses

Experimental feeds and individual prawn whole bodies were freeze-dried (Dynavac, FD3); moisture loss was determined by drying and weighing to a constant weight. Chemical composition of feeds and whole bodies were analysed in duplicate at the Nutrition Laboratory (University of Tasmania, Launceston, Tasmania). Feed samples were homogenised to a fine powder using a bench-top hammer mill (Culatti micro hammer-cutter mill, MFC), before being wrapped in aluminium foil and placed in an airtight plastic bag at -20°C until required. Whole prawns were ground to fine powder using a mortar and pestle and homogenised samples stored under the same conditions.

Crude protein analysis was conducted using the Kjeldahl method (FOSS Kjeltec™ 8100, crude protein was calculated as N x 6.25), crude lipid (Bligh and Dyer, 1959), gross energy by bomb calorimetry (Gallenkamp Autobomb, CAB101) and ash by combustion (SEM 102C muffle furnace) at 600°C for 2 h, using standard laboratory methods in accordance with AOAC (1995).

Moisture content of feeds was determined by drying a 2 g sample of feed in triplicate at 135°C to a constant weight, moisture content was calculated as the weight lost after drying.

All analyses for the determination of fucoidan, polyphenol and sulfate concentrations were done by determining fucose concentration in freeze-dried prawn feeds and back-calculating to determine final fucoidan concentration in feeds. Total carbohydrate content was determined by spectrophotometric analysis of the hydrolysed compound in the presence of phenol, a proprietary method of Marinova Pty Ltd based on Dubois et al. (1956). Sulfate content was analysed spectrophotometrically using the $BaSO_4$ precipitation method ($BaCl_2$ in gelatin), based on Dodgson (1961) and Dodgson and Price (1962). Analysis of polyphenol content was determined

spectrophotometrically using a modified method based on the Folin-Ciocalteu reagent (phosphomolybdate/phosphotungstate) (Zoecklein et al., 1999; Jimenez-Escrig et al., 2001; Zhang et al., 2006). The carbohydrate profile was determined using a GC-based method for the accurate determination of individual monosaccharide ratios in Marinova samples, using acetylated alditol derivatives in the hydrolysed samples (Morvai-Vitanyi et al., 1993). Molecular weight profiles were determined by gel permeation chromatography, with the aid of a size exclusion column, reported relative to Dextran standards.

3.7 Calculations

Prawn days (P.d) was calculated as:

$$P.d\ (\text{mean prawn.days}^{-1}) = (d._{feeding} / d._{experiment})$$

Where: $d._{feeding}$ = cumulative number of days where prawns were alive and feeding, $d._{experiment}$ = duration of experiment in days. P.d is the mean number of surviving prawns per day for calculating daily FI and FER to standardise data due to mortality.

Water stability was calculated as:

$$\text{Water stability (\%)} = (FW_{dry}\ (g) / (IW_{wet}\ (g) \times \%DM / 100)) \times 100$$

Where: FW_{dry} = final dry weight after immersion, IW_{wet} = initial weight of pellet before drying and %DM = percentage dry matter in pellets.

Feed intake (FI) (g DM^{-1} Pd^{-1}) was calculated as total feed consumed (g dry matter) less uneaten feed and dry matter losses due to leaching.

$$FI = (\text{consumed } W_{feed\ DM}\ (g) - M_{loss}) \times W_{stability} / 100) \times P.d^{-1}$$

Where: $W_{feed\ DM}$ = total dry weight of feed consumed (g), M_{loss} = pellet moisture loss (g), $W_{stability}$ = pellet dry matter loss, P.d = prawn.days^{-1} where mean number of surviving prawns per day.

Weight gain was calculated as:

Weight gain (g) = (FW_{wet} (g) – IW_{wet} (g))

Where: FW_{wet} = final wet weight, IW_{wet} = initial wet weight.

Specific growth rate (SGR) was calculated as:

SGR (% d^{-1}) = ((Ln (W_{final}) – Ln ($W_{initial}$)) / d. x 100

Where: W_{final} = mean final wet weight (g), $W_{initial}$ = mean initial wet weight (g), d. = no. of days (Ricker, 1979).

Average weekly gain (AWG) was calculated as:

AWG = (W_{final} (g) – $W_{initial}$ (g)) / wk

Where: W_{final} = mean final wet weight (g), $W_{initial}$ = mean initial wet weight (g), wk = duration of experiment in weeks.

Feed efficiency ratio (FER) was calculated as:

FER = (meanWT_W (g) / FI (g)) x 100)

Where: WT_W = mean wet weight gain (g), FI = feed intake (g DM mean Pd^{-1}) after adjustments for uneaten feed and leaching.

Total haemocyte count (THC cells.ml^{-1}) was calculated as:

THC = (MHC x 2.5 x 10^5) x DF

Where: MHC = mean no. of haemocytes per medium square of haemocytometer, 2.5 x 10^5 cells.ml = conversion factor, changing millimetres to millilitres, DF = dilution factor of the addition of fixative and stain (Sritunyalucksana et al., 2005).

Differential haemocyte count (DHC %) was calculated as:

DHC = (GH / total haemocytes counted) x 100

Where: GH = total number of small and large granular haemocytes.

Granular haemocyte count (GHC cells.ml^{-1}) was calculated as:

GHC = (DHC x THC)

Where: DHC = mean number of small and large granular haemocytes as a percentage, THC = mean number of haemocytes per ml of haemolymph (Sritunyalucksana et al., 2005).

3.8 Statistical analyses

Tanks were considered a replicate unit for growth and survival data, while individual prawns within tanks were considered as replicate units for measured immune response data. Initial baseline data were not included in statistics. Test of equal variances were assessed using Levene's test of equality and residual plots.

All statistical analysis and data transformations were conducted using IBM SPSS STATISTICS (version 21). Means were compared by one-way Analysis of Variance (ANOVA) for comparisons between growth, feed and chemical composition data, and pooled data for comparisons between metabolic parameters, when no interaction between feed treatments and stressor treatments were significant. Interactions between treatment and stressor for immune responses were compared by two-way Analysis of Variance (ANOVA). Results were considered significant if $P < 0.05$ using a Tukey's HSD test. To determine whether a tank effect was present for immune responses a three factor cross mixed model ANOVA (model =Treatment + Stressor + Treatment*Stressor + Tank(Treatment*Stressor) was conducted. Survival percentage data were arcsine transformed prior to a one-way ANOVA analysis. Tables and figures were constructed using Microsoft Office Excel 2010 and Sigma Plot, version 11.0 (Systat Software, Inc).

3.9 Results

3.9.1 Feed stability

There was no significant difference in mean pellet stability after immersion in culture water for 1 h, pellet stability ranged from 90.87% (basal) up to 92.41% (FV90) (F = 3.281, df = 3, 12, *P* = 0.08) (Fig.3.1).

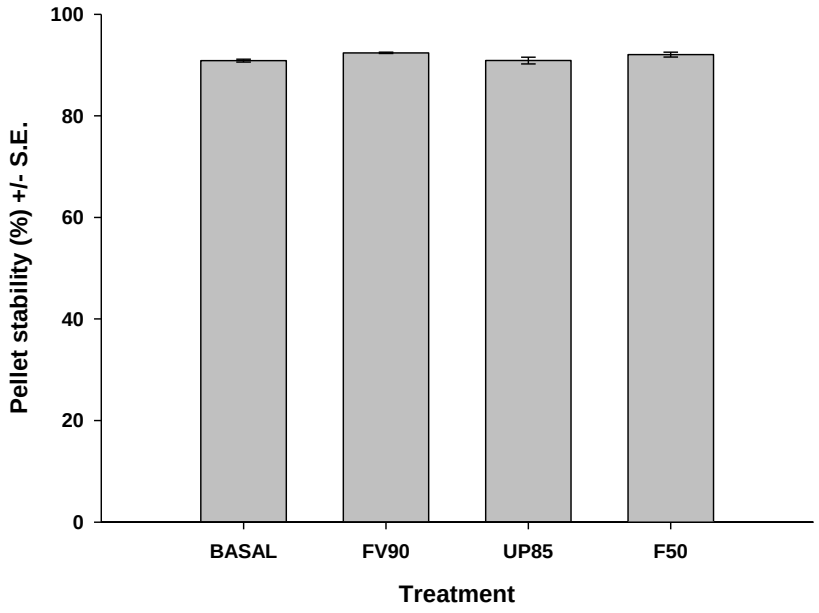

Figure 3.1. Mean pellet water stability (%) ± S.E. for feeds after 1 h immersion in experimental tanks.

3.9.2 Growth performance

At the start of the trial the initial mean prawn weights (6.27 ± 0.20 g) were not significantly different (F = 0.020, df = 3, 20, *P* = 0.996) between tanks (Table 3.2). At the conclusion of the growth trial mean prawn weights (pooled mean 11.19 ± 0.50 g) were not significantly different between feed treatments (F = 0.230, df = 3, 20, *P* = 0.875). Daily pooled mean prawn growth was 2.78% per day (F = 0.125, df = 3, 20, *P* = 0.944) and mean weight gain was similar (F =

0.241, df = 3, 20, *P* = 0.867) for prawns fed all treatments, ranging from 77.07% for prawns fed the basal treatment up to 81.19% for prawns fed F50 (Table 3.2). The feed efficiency ratio of prawns was not significantly different between treatments, ranging from 41.19 ± 2.63 (UP85) to 49.65 ± 2.79 (FV90) (F = 1.350, df = 3, 20, *P* = 0.287) (Table 3.2). The mean survival rate of prawns was similar (F = 0.230, df = 3, 20, *P* = 0.874) across all feed treatments after 21 d, ranging from 89.29% (FV90 and UP85) to 92.86% (basal and F50) (Table 3.2).

3.9.3 Whole body chemical composition

The chemical composition of prawns based on a wet weight was not influenced by feed treatment after feeding for 21 days (Table 3.3). Whole body dry matter of prawns ranged from 267.54 $g.kg^{-1}$ for prawns fed the basal feed up to 275.38 $g.kg^{-1}$ for prawns fed the FV90 feeds and there was no significant difference (F = 0.251, df = 3, 44, *P* = 0.860) (Table 3.3). The mean crude protein content of prawns was not significantly different between feed treatments (F = 0.676, df = 3, 44, *P* = 0.571), ranging from 196.22 ± 4.45 (basal) to 205.37 ± 6.32 $g.kg^{-1}$ (FV90) (Table 3.3). The mean crude lipid content of prawns was not significantly different between feed treatments (F = 0.350, df = 3, 44, *P* = 0.790), ranging from 21.06 ± 1.07 (F50) to 22.48 ± 1.17 $g.kg^{-1}$ for prawns fed UP85 (Table 3.3). Mean whole body gross energy of prawns was not significantly different between feed treatments (F = 0.284, df = 3, 44, *P* = 0.837), ranging from 4.99 ± 0.12 to 5.17 ± 0.15 $MJ.kg^{-1}$ for prawns fed the basal and FV90 treatments respectively (Table 3.3).

Table 3.2. Mean growth performance indices and survival (n = 6, ± S.E.) of sub-adult *Penaeus monodon* fed feeds containing different sources of fucoidan after 21 d, (df = 3, 20).

	Units	Feeds				One-way ANOVA	
		BASAL	**FV90**	**UP85**	**F50**	**F-value**	***P***
Initial weight	g	6.32 ± 0.51	6.19 ± 0.25	6.31 ± 0.57	6.25 ± 0.29	0.020	0.996
Final weight	g	11.04 ± 0.52	11.14 ± 0.35	11.30 ± 0.61	11.31 ± 0.53	0.230	0.875
Feed intake[1]	g DM Pd^{-1}	4.37 ± 0.33	4.60 ± 0.42	5.19 ± 0.54	5.12 ± 0.33	0.909	0.454
Weight gain[2]	g	4.72 ± 0.64	4.95 ± 0.48	5.00 ± 0.71	5.06 ± 1.03	0.241	0.867
Specific growth rate[3]	% d^{-1}	2.70 ± 0.19	2.80 ± 0.08	2.81 ± 0.14	2.81 ± 0.16	0.125	0.944
Average weekly gain[4]	g wk^{-1}	1.57 ± 0.09	1.65 ± 0.06	1.68 ± 0.08	1.68 ± 0.14	0.300	0.825
Feed efficiency ratio[5]	%	46.92 ± 3.08	49.65 ± 2.79	41.19 ± 2.63	45.93 ± 3.55	1.350	0.287
Survival	%	92.86 ± 4.12	89.29 ± 6.84	89.29 ± 3.57	92.86 ± 7.14	0.230	0.874

Feeds contain: BASAL = Control, FV90 = Maritech® *F. vesiculosus*, UP85 = Maritech® *U. pinnatifida*, F50 = Fucorich® *U. pinnatifida* extract.

[1]Feed intake (g DM Pd^{-1}) = (dry matter of feed fed, after adjustments for uneaten feed and leaching) / Prawn days of feeding (Pd^{-1})).

[2]Weight gain (g) = final wet weight – initial wet weight.

[3]Specific growth rate (% d^{-1}) = ln(final wt.) – ln(initial wt.) x 100 / days of experiment.

[4]Average weekly gain (g wk^{-1}) = (wet weight final – wet weight initial / weeks of experiment.

[5]Feed efficiency ratio (%) = (mean wet weight gain (g) / feed intake (g DM mean Pd^{-1}) after adjustments for uneaten feed and leaching) x 100.

$P < 0.05$ was considered significant.

Table 3.3. Mean chemical composition (g.kg^{-1} wet weight ± S.E.) of sub-adult *Penaeus monodon* fed feeds containing different sources of fucoidan after 21 d, (df = 3, 44, n = 12).

	Feeds					One-way ANOVA		
	INITIAL	**BASAL**	**FV90**	**UP85**	**F50**	**F-value**	***P***	***P***
Dry matter	256.45 ± 5.25	267.54 ± 5.40	275.38 ± 7.93	273.39 ± 4.80	270.36 ± 8.58	0.251	0.860	
Crude protein	185.65 ± 4.41	196.22 ± 4.45	205.37 ± 6.32	203.96 ± 5.52	198.45 ± 4.74	0.676	0.571	
Crude lipid	17.59 ± 1.16	21.26 ± 1.04	21.64 ± 0.95	22.48 ± 1.17	21.06 ± 1.07	0.350	0.790	
Gross energy (MJ.kg^{-1})	4.66 ± 0.10	4.99 ± 0.12	5.17 ± 0.15	5.11 ± 0.10	5.08 ± 0.16	0.284	0.837	

Feeds contain: BASAL = Control, FV90 = Maritech® *F. vesiculosus*, UP85 = Maritech® *U. pinnatifida*, F50 = Fucorich® *U. pinnatifida* extract.
Initial baseline data (n = 6) are not included in statistics.
$P < 0.05$ was considered significant.

3.9.4 Immune response

There was no interaction between feed treatments and stressor treatments for THC in prawns when assessed by two-way ANOVA (F = 0.946, df = 3, 64, *P* = 0.424), therefore THC data were pooled by non-stress and stress treatments. Prawns subjected to lower water temperature and salinity had a significantly lower mean THC (F = 15.067, df = 1, 70, *P* <0.001), which was on average 18.59% lower than for the prawns held at the optimum water parameters (Figure 3.2).

Feed treatments had no effect on the GHC in prawns and there was no interaction between feed treatment and stressor treatment (F = 1.282, df = 3, 64, *P* = 0.288) (Figure 3.3). When GHC were pooled for non-stress and stress treatments there was a significant decrease in the number of granular haemocytes in prawns subjected to lower water temperature and salinity (F = 5.525, df = 1, 70, *P* = 0.022). On average the stressed prawns had a 17.62% reduction in the number of granular haemocytes (Figure 3.4).

There were no statistical effect of feed and stressor treatments on the percentage (DHC) of granular haemocytes (Figure 3.5, (F = 1.191, df = 3, 67, *P* = 0.320)). There was no significant difference (F = 0.609, df = 3, 64, *P* = 0.612) in phenoloxidase activity (PO activity) for prawns fed different feed treatments and subjected to stress or non-stress conditions (Figure 3.6). Data were assessed for tank and sampling time effects using a three-way mixed model ANOVA and a significant sampling time effect was determined (F = 4.775, df = 4, 12.109, *P* = 0.015), therefore no further comparison of data could be made.

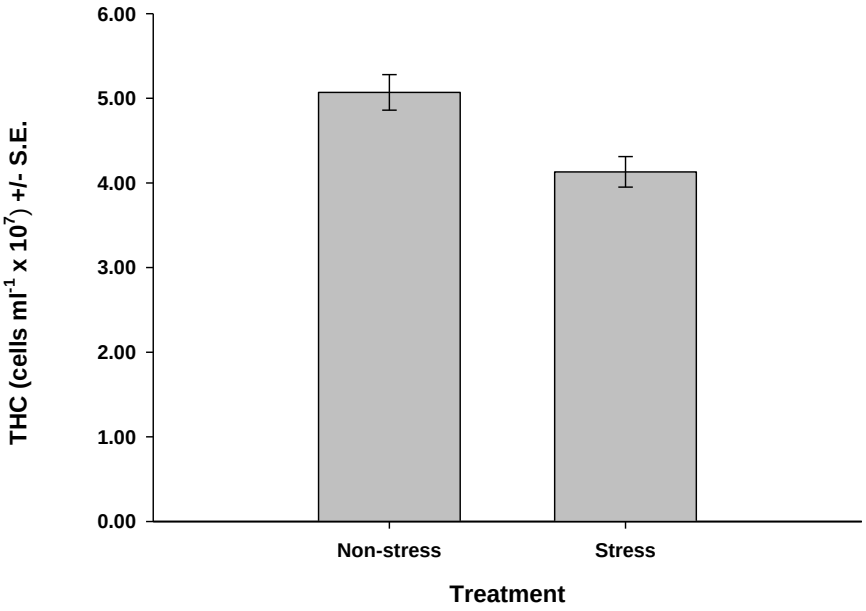

Figure 3.2. Mean total haemocyte counts (THC) (cells ml^{-1} x 10^7 ± S.E.) of *Penaeus monodon*, diets pooled for non-stress and stress treatments (F = 15.067, df = 1, 70, *P* <0.001). *P* < 0.05 was considered significant.

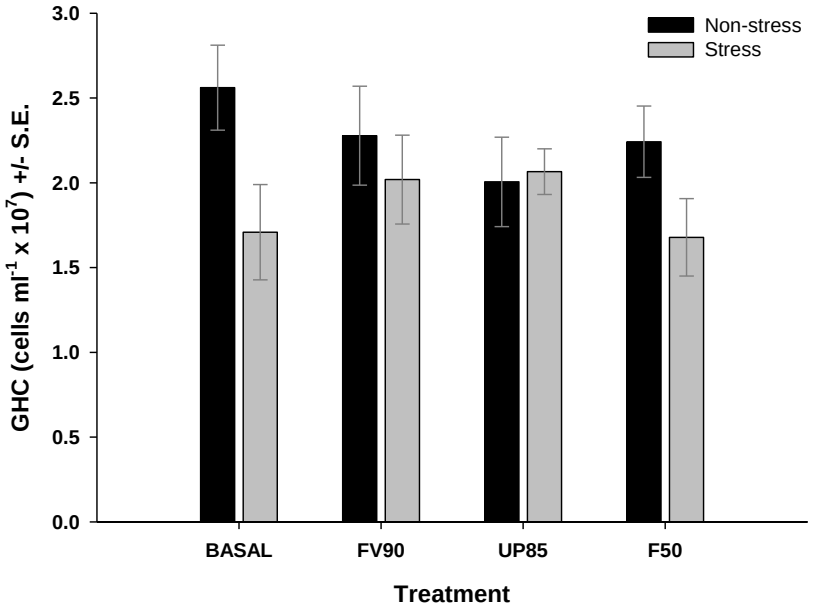

Figure 3.3. Mean granular haemocyte counts (GHC) (cells ml^{-1} x 10^7 ± S.E.) of *Penaeus monodon* fed on different feed treatments and after stressor treatments (F = 1.282, df = 3, 64, *P* = 0.288). *P* < 0.05 was considered significant.

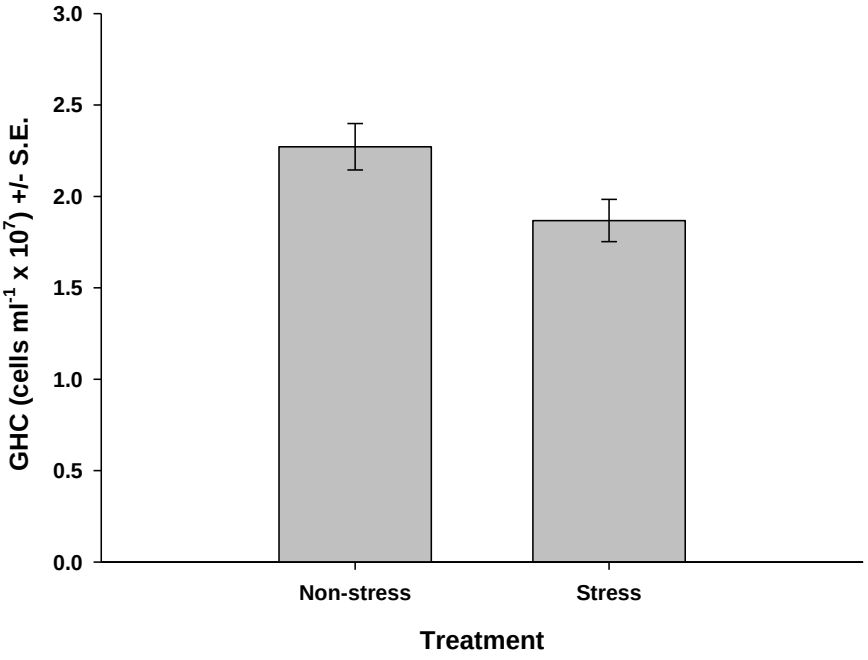

Figure 3.4. Mean granular haemocyte counts (GHC) (cells ml^{-1} x 10^7 ± S.E.) of *Penaeus monodon* pooled for non-stress and stress treatments (F = 5.525, df = 1, 70, $P = 0.022$). $P < 0.05$ was considered significant.

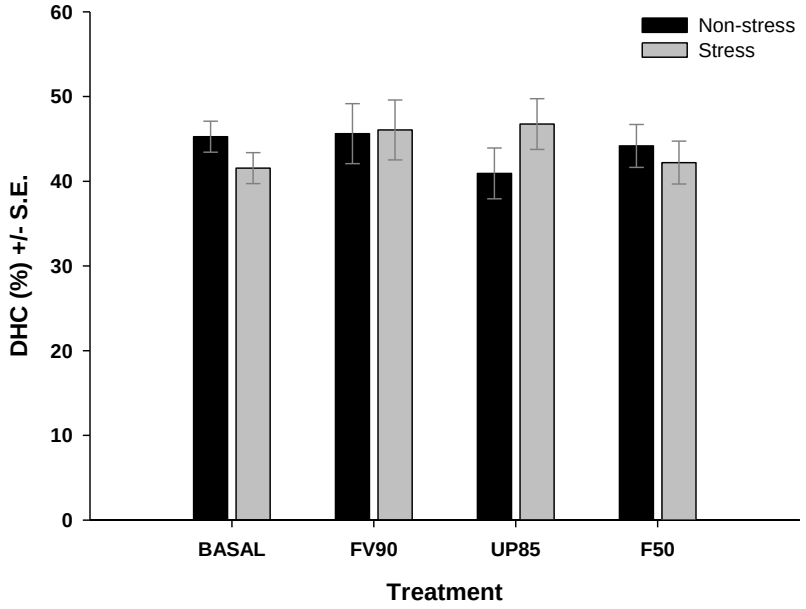

Figure 3.5. Mean differential haemocyte counts (DHC) (% ± S.E.) of *Penaeus monodon* fed on different feed treatments and after stressor treatments (F = 1.191, df = 3, 67, *P* = 0.320). *P* < 0.05 was considered significant.

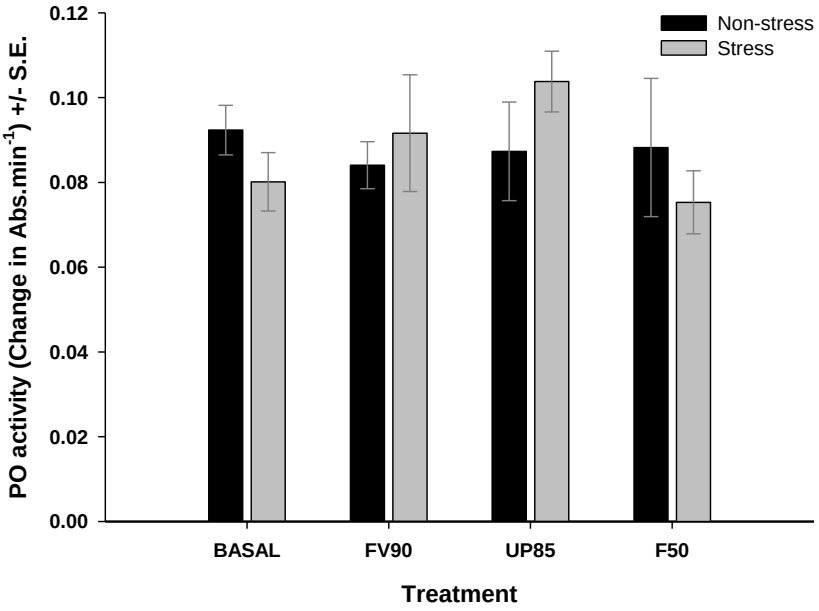

Figure 3.6. Mean phenoloxidase activity (PO activity) (Δ in Abs.min^{-1} ± S.E.) of *Penaeus monodon* for the interaction of feed treatment and non-stress/stress treatments (F = 0.609, df = 3, 64, *P* = 0.612). *P* < 0.05 was considered significant.

3.10 Discussion

A combined drop in water temperature and salinity had a significant impact on the number of circulating haemocytes within the haemolymph of *P. monodon*. Mean total haemocyte numbers (THC) dropped by 9.4 x 10^6 cells ml^{-1} or 18.59% for prawns exposed to a decrease in water temperature and salinity. Furthermore, granular haemocyte counts (small and large granular haemocytes) followed a similar decreasing trend; there was a mean reduction of 17.62% in granular haemocytes per ml of haemolymph. This drop in haemocyte numbers in prawns held under stress conditions suggests that the changing environmental conditions has elicited a stress response, and therefore supressing the immune system, the immunosuppression manifested itself as a decrease in haemocyte numbers within the haemolymph. Reductions in haemocyte numbers have been widely documented during periods of adverse environmental conditions, resulting in impaired immune function of prawns and or succumbing to disease infections caused by viral pathogens (Persson et al., 1987; Le Moullac and Haffner, 2000; Perazzolo et al., 2002; Liu et al., 2006; Joseph and Philip, 2007). Significant reductions in haemocyte numbers have been reported in marine prawns from acute changes in water temperature and salinity (Le Moullac and Haffner, 2000; de la Vega et al., 2006; Wang and Chen, 2006; Pan et al., 2008), hypoxia (Direkbusarakom and Danayadol, 1998; Le Moullac et al., 1998), changing water chemistry (Li and Chen, 2008), toxic substances (Smith et al., 1995) and viral and bacterial infections (Mikulski et al., 2000; Joseph and Philip, 2007; Yeh et al., 2010).

Shrimp (*Crangon crangon*) exposed to contaminated dredge soil, displayed a decrease in THC; this decrease was explained by an increase in haemolymph volume and not to a decrease in total cell numbers (Smith et al., 1995). However, when prawns were infected with white spot syndrome virus (WSSV); there was a decrease in the number of haemocytes, which were thought

to be related to haemocytic accumulations at injection points, due to these haemocytes being phagocytosed (Ratcliffe and Rowley, 1979; Song et al., 2003). While in the current study the decrease in haemocyte numbers may be explained by a reduction in haemolymph proteins rather than an accumulation of haemocytes within a specific location within the haemocoel, as prawns were not challenged with a pathogen, rather an environmental stressor (Perazzolo et al., 2002).

In the current study there was no difference in PO activity after feeding prawns on control and fucoidan feeds for 21 d, and after the decrease in water temperature and salinity. While this may differ from the literature (Perazzolo et al., 2002; Joseph and Philip, 2007; Traifalgar et al., 2010; Kitikiew et al., 2013), a high individual variation may partly explain the lack of any differences, in particularly between stress and non-stress treatments. Another explanation is that samples were staggered over a 6 day period to ensure processing of individual samples were not compromised due to clotting factors. When PO activity data were analysed by three-way ANOVA, a statistically significant sampling time effect was determined, therefore some days samples resulted in low PO activity values while other days had high values across all treatments. This may have been caused by a number of factors, such as processing time or sample storage temperature.

In contrast to the findings of this study, when white shrimp (*Litopenaeus vannamei*) were fed diets containing fucoidan (1 $g.kg^{-1}$) there was a significant increase in PO activity after 14 and 21 days (Kitikiew et al., 2013). For prawns fed the same diet, there was a significant increase in phagocytic activity and clearance efficiency against *Vibrio alginolyticus*. Therefore, dietary fucoidan has demonstrated to provoke innate immunity in penaeid prawns by improving degranulation in haemocytes and activating the prophenoloxidase system which enhances the immune response and resistance against *V. alginolyticus* (Kitikiew et al., 2013).

Decreasing water salinity from 33‰ to 22 and 13‰ respectively in *Farfantepenaeus paulensis* resulted in a reduction in THC and PO activity (Perazzolo et al., 2002). Prawns held under optimal environmental conditions also experienced significant decreases in PO activity when sampled at day 21 and 28. This pattern of decreasing PO activity over time may also explain results in the current research over the 21 days of experimentation. However to further explore the effect of long term holding and experimentation on PO activity a series of sampling events are required to determine immune response over time.

Prawns were fed to satiation during the feeding trial and fresh feed was delivered four times each day. There was no indication that feeding *P. monodon* fucoidan at 1 $g.kg^{-1}$ for 21 days caused any negative effects on growth, as FI, SGR, AWG and final prawn weights were not significantly different for prawns fed all feed treatments, in fact prawns fed fucoidan performed equally to those fed commercial feeds. When *Marsupenaeus japonicus* were fed diets containing 0.5 and 1 $g.kg^{-1}$ fucoidan, the results demonstrated an increase in growth performance with a decrease in feed conversion ratio after 56 days (Traifalgar et al., 2010). The authors also determined that prawns fed fucoidan had increased carcass lipid and protein retention. While these beneficial effects were due to increased lipid accretion in muscle tissue and efficient utilisation of energy stores as previously determined in studies where algal meals were fed to red sea bream (*Pagrus major*) (Yone et al., 1986; Nakagawa, 1997). However, in the current study the whole body chemical composition was not significantly different at the conclusion of the experiment. Therefore, prawns fed fucoidan treatments did not show enhanced crude protein or lipid accretion as previously reported by Traifalgar et al. (2010). These differences may be attributed to the feeding trial duration, which was 2.6 times greater in the study by Traifalgar et al. (2010).

However, in this study all prawns had equivalent energy reserves and condition prior to the temperature and salinity stressors.

Average specific growth rate for fucoidan fed *P. monodon* in this study was 2.81% per day compared to 1.9 to 2.06% per day when *M. japonicus* were fed 0.5 and 1 $g.kg^{-1}$ (Traifalgar et al., 2010). Furthermore, feed efficiency in the current experiment ranged from 41.19% (F50) to 49.65% for prawns fed FV90; FER is corrected for uneaten feed, water leaching and mortalities. In comparison the feed conversion ratio (FCR) for prawns fed fucoidan in the study by Traifalgar et al. (2010) was 1.8, which is higher than the average FCR equivalent in the current research of 1.10. This may have restricted potential growth enhancing benefits as larger prawns grow at a much slower rate than small prawns. In fact feeding prawns a diet containing fucoidan at early larval stage may provide better growth performance, due to the requirement for high quality nutrition at early life stages (D'Abramo and Castell, 1997; Traifalgar et al., 2012).

In the present study the duration of the feeding trial was 21 days; this duration was determined from similar published studies that assessed changes in haemocyte mediated immune responses after feeding immunostimulant products (Chang et al., 2003; Kitikiew et al., 2013; Peraza-Gomez et al., 2014). To ensure enough haemolymph volume was available for each immune response measurement (THC, DHC & PO activity); prawns with a mean weight of 6 g were selected for the experiment. Therefore the starting weight of prawns in this experiment was much larger than in the study conducted by Traifalgar et al. (2010). Prior to transferring prawns to stress and non-stress treatments, each animal was moult staged to ensure all animals were in intermoult, this process was conducted a second time prior to haemolymph removal for measuring immune responses. Physical handling and exposure to air for 10 min can cause stress and an increase in haemolymph glucose concentration (Hall and Van Ham, 1998). While

handling is an important process of any sampling event all prawns were handled in the same manner and duration therefore standardising any effect this process may have had on individuals.

3.11 Conclusion

In this study the application used for testing fucoidan in *P. monodon* feeds have demonstrated little evidence for increased growth rates and immune responses. It is possible that this was caused by the dose of fucoidan used, and the duration of the feeding study. Using the current dose of fucoidan and protocol there were no effects of fucoidan on growth or immune response compared to prawns fed the commercial feed treatment. There were also no negative effects on feed intake or survival of *P. monodon*. There was a consistent decrease in THC and GHC with environmental change.

Future studies to assess fucoidan in *P. monodon* feeds should explore the potential immunostimulant benefits of fucoidan after an environmental challenge and how environmental stress impacts upon the aerobic metabolism; by measuring oxygen consumption under varying environmental conditions.

3.12 References

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Metabolic capacity and immune response of *Penaeus monodon* fed fucoidan, after a combined drop in water temperature and salinity

CHAPTER IV

4.1 Abstract

The physiological stress caused by environmental changes can have a negative influence on both, the aerobic metabolism ($\dot{M}o_2$) and immune system of prawns. Individual experiments were conducted on juvenile and sub-adult *Penaeus monodon*. In each experiment prawns were fed feeds containing either 1 $g.kg^{-1}$ fucoidan (FV90) or a control feed (basal) for approximately 21 d, prior to conducting an environmental stress test. Juvenile prawns were subjected to an acute decrease in water temperature from 30-24°C and salinity from 40-20‰ (stress treatment) over 7 h, before measuring $\dot{M}o_2$ which includes: standard metabolic rate (SMR), routine metabolic rate (RMR), maximal metabolic rate (MMR), active metabolic rate (AMR) and aerobic scope (A-SCOPE), using an intermittent flow static respirometry system during a 20 h period. At the conclusion of $\dot{M}o_2$ measurements pleopod samples were taken to determine Gill-Associated Virus (GAV) concentrations. Sub-adult prawns were subjected to the same environmental stress test, over a longer period (72 h) prior to $\dot{M}o_2$ measurements, to simulate chronic stress. A non-stress control (30°C and 40‰) for both feed treatments was also tested with sub-adult prawns. For sub-adult prawns, haemolymph was sampled to determine the total haemocyte count (THC), granular haemocyte count (GHC) and phenoloxidase activity (PO activity), and pleopods were sampled for GAV concentration.

Juvenile *P. monodon* fed FV90 had a significantly higher AMR compared to prawns fed the basal feed. There were no significant differences in SMR, RMR, MMR or A-SCOPE for prawns fed either feed treatment. Juvenile prawns fed each feed treatment tested positive for GAV; however there was no difference in viral concentration.

When sub-adult prawns were fed the basal and FV90 treatments there were no significant interactions between feed treatments and stressor treatments for all $\dot{M}o_2$ measurements. When data were pooled by stressor, the non-stress group had significantly higher $\dot{M}o_2$ rates than prawns subjected to the stress conditions.

There were no significant interactions of feed and stressor treatments on THC, GHC and PO activity. When data were pooled within stress and non-stress groups for immune response analyses (THC, GHC and PO activity), prawns held under stress conditions had significantly higher values. On average stressed prawns had 25% more total haemocytes and 39% more granular haemocytes than prawns held under optimal conditions and PO activity of stressed prawns was on average 61.7% higher PO activity than prawns under optimal conditions. All prawns tested positive for GAV, however there were no significant differences between feed treatments or stressor treatments. Sub-adult prawns had on average 80 times more GAV copies/ng of total RNA.

Fucoidan failed to influence prawn survival and incidence of opportunistic GAV infection. Under acute stress sub-adult prawns had reduced aerobic capacity and exhibited a greater immune response in terms of THC, GHC and PO activity. Fucoidan had little effect on $\dot{M}o_2$ except increased AMR in juvenile prawns fed fucoidan.

4.2 Introduction

Penaeus monodon are a euryhaline and poikilothermic species and can inhabit a vast range of salinities and water temperatures, however acute changes in salinity and temperature can cause stress (Rajesh et al., 2012). Such combined changes in environmental conditions are commonly experienced during the monsoonal season in tropical aquaculture, where heavy rainfall results in a rapid decline of prawn culture water temperature and salinity. These rapid changes in environmental conditions can result in prawn mortality and a significant reduction on aquaculture productivity (de la Vega et al., 2006). The physiological stress caused by changes in the environmental conditions can have negative influences on both the aerobic metabolism (oxygen consumption rate, $\dot{M}o_2$) and immune system of prawns (Priede, 1985; de la Vega et al., 2006; Sokolova et al., 2012). However, there is little information on the effects of immunostimulants on metabolic response and capacity of prawns to environmental stress.

Aerobic metabolism of aquatic animals can be used as an indirect measure of spontaneous energy consumption and can be measured at differing states of activity. The standard metabolic rate (SMR) represents the minimal $\dot{M}o_2$ of the intact organism at rest, routine metabolic rate (RMR) is the metabolism of non-stressed animals under random activity and the active metabolic rate (AMR) represents the maximum metabolism which is commonly measured immediately after exhaustion in crustaceans (Ern et al., 2014; Fitzgibbon et al., 2014). The aerobic scope is the difference between the SMR and AMR and reflects the aerobic capacity for energy production to fuel physiological function beyond maintenance requirements (Fry and Hart, 1948; Sokolova et al., 2012). The role of aerobic scope in the stress tolerance of aquatic organism has been studied in relation to numerous environmental factors including, temperature, dissolved oxygen and salinity and the combinations thereof. There is a strong body of evidence to suggest a direct link between the ability of aquatic

organism to maintain aerobic scope and physiological fitness in response to environmental stress (Frederich and Portner, 2000; Sokolova et al., 2012). At environmental extremes, aerobic scope of aquatic organism has been shown to decline due to an inability of the cardiorespiratory system to supply oxygen to metabolically active tissues (Frederich and Portner, 2000; Portner, 2010; Sokolova et al., 2012). Whilst it is the combined influence of temperature and salinity stress on cultured shrimp SMR and RMR has been extensively examined (Spanopoulos-Hernandez et al., 2005). By measuring the aerobic scope, it may be possible to determine how well prawns perform under changing environmental conditions, with an increase in aerobic scope providing a higher capacity for prawns to tolerate a stressful event (Chen and Nan, 1993), therefore increasing survival and growth or reducing the stress on the animal (Bray et al., 1994; Villarreal et al., 2003).

Little is known about the effect of dietary immunostimulant on aerobic capacity and corresponding stress tolerance of crustaceans. A previous study found that the immunostimulation with soluble polysaccharide extract from the algae *Macrocystis pyrifera*, haltered metabolic depression of white shrimp infected with *Vibrio campbellii* (Sanchez-Campos et al., 2010). Metabolic depression is a reduction in aerobic metabolism during disease infection and is thought to be a due to the immune response impairing normal metabolic function (Burnett et al., 2006; Scholnick et al., 2006; Thibodeaux et al., 2009). This reduction in metabolism likely negatively affects overall physiological performance and ability to cope with environmental stress. Further research is required to determine if immunostimulants, such as fucoidan, may act to reduce the influence of metabolic depression and be beneficial by supporting physiological capacity to maintain aerobic scope under environmental stress.

An acute change in salinity or temperature also has a significant effect on immune parameters (Joseph and Philip, 2007; Pan et al., 2008) including a decrease in total numbers of haemocytes (THC), granular haemocytes (GHC) and phenoloxidase activity (PO activity) (Pan et al., 2005; Wang and Chen, 2006; Joseph and Philip, 2007). A decrease in salinity by 15‰ reduced the total haemocyte counts (THC) and phenoloxidase activity (PO activity) of *P. monodon* (Joseph and Philip, 2007). Similarly, when salinity decreased from 30 to 15‰ a reduction in THC and PO activity of *Litopenaeus vannamei* was observed, before returning to normal baseline values at 6 days post-stress (Pan et al., 2005). Environmental stress can suppress immune capacity and increase the susceptibility to pathogens, resulting in higher mortalities (Smith et al., 1995; Le Moullac and Haffner, 2000).

To counteract disease and mortality caused by pathogens, the use of immunostimulant products such as fucoidan in prawn feeds has shown to be beneficial in *Litopenaeus vannamei* (Kitikiew et al., 2013), *Marsupenaeus japonicus* (Takahashi et al., 1998; Traifalgar et al., 2010; Traifalgar et al., 2012), *P. monodon* (Chotigeat et al., 2004; Immanuel et al., 2012). On this basis, fucoidan can increase survival to pathogens; this may also provide some benefit under fluctuating environmental conditions, for supporting immune function (Traifalgar et al., 2010; Immanuel et al., 2012; Kitikiew et al., 2013). However, it is not clear whether immunostimulant products such as fucoidan can influence the A-SCOPE in prawns or enhance $\dot{M}o_2$ under suboptimal or acute changes in environmental conditions. During the process of commercial prawn farming many factors affect the overall welfare of the animal, including varying pond water conditions within normal seasonal fluctuations and exposure to viral and bacterial pathogens of differing concentration and virulence. Further understanding of how $\dot{M}o_2$ are influenced by changing environmental conditions will provide an insight into of how immunostimulant function to improving disease resilience of prawns.

The aims of this study were:

- To determine the effects of acute and chronic environmental stress exposure on the aerobic metabolism of *P. monodon*.
- To determine whether fucoidan enhances aerobic metabolic rates in juvenile of *P. monodon* under stressful conditions.
- To determine the effect of fucoidan on aerobic metabolic rates in sub-adult *P. monodon* under optimal and stressful conditions.
- To measure immune responses (THC, DHC and PO activity) of *P. monodon* after a combined decrease in water temperature and salinity, in comparison to non-stress control groups fed feeds with and without fucoidan.

4.3 Materials and methods

4.3.1 Prawn stock

Ten thousand 14 d old black tiger prawn (*P. monodon*) postlarvae (PL) were obtained from Rocky Point Prawn Farm, Gold Coast, Queensland, Australia. Postlarvae were transported by air to the University of Tasmania, Launceston, Tasmania where they were held under Quarantine (Special Authority Number, SA 12-21) in two raceway tanks within a recirculating seawater system. Postlarvae were fed a mixture of live *Artemia* spp. (INVE, premium) and commercial prawn crumble (Prawn Starter Enhance MR, Ridley Aqua-Feed) three times daily to satiation until PL 28. Thereafter, PL were fed to satiation twice daily on a commercial prawn feed at 0800 and 1700 by hand, with an additional 4 feeds (2000, 2300, 0300 and 0500) from 2 digital automatic feeders (Automatic Fish Feeder, FF3). Postlarvae were maintained under optimum environmental conditions (salinity: 34-37‰; photoperiod: 12:12 light dark cycle; temperature: 28-30°C) and water quality parameters (NH_3: < 0.5 ppm; NO_2: < 0.5 ppm; NO_3: < 20 ppm and pH: 8.2), measured using a saltwater master test kit (API™).

Two separate experiments were conducted to assess the aerobic metabolic rates of juvenile and sub-adult *P.* monodon. Juvenile prawns were on-grown to approximately 0.5 g (PL64), while sub-adult prawns from the same cohort were on-grown to mean weight = 17.05 ± 0.61 g, prior to an acclimation period in experimental tanks.

4.3.2 Experimental system

Growth trials were conducted in a replicated recirculating seawater system comprising of twenty four 118 L rectangular (surface area = 0.43 m^2) high density polypropylene tanks. Water quality was maintained by biofiltration, 18 W ultraviolet (UV) sterilization (Emperor Aquatic's) and solids removal using a dacron screen. Water was heated by an 8.1 kW reverse

cycle air conditioner (Mitsubishi Electric, MUZ-GA71VA) and a submerged emersion immersion water heater (Aqua One, 60 W) which maintained temperatures at 30^{o}C. Salinity was maintained at 40‰ through the addition of de-chlorinated tap water or pool salt (Cheetham Salt, Mermaid Finest, Australia) and was measured daily using a salinity refractometer (IWAKI, TD5Q8). Aeration was provided by individual air-stones in each tank which maintained dissolved oxygen levels above 100% saturation. Tanks were fitted with grey PVC hinged tank covers and tank outlets with fibreglass flyscreens (1 mm²) to prevent the prawns from escaping. The average flow rate per tank was 1.9 L/min, exchanging tank volume every 55 min. Light intensity in the rooms was below 0.33 µmol photons PAR m^{-2} s^{-1} (Biospherical Instruments INC) and red cellophane was used to cover lights so prawns were not disturbed during feeding and maintenance. One hide made from bunched strips of fibreglass mesh material (1 mm²), suspended under polystyrene blocks was placed in each tank to reduce cannibalism. During experimentation prawns were fed four times per day (07:30, 12:00, 16:00 and 19:30) to satiation. All uneaten pellets, faeces and exuvia were siphoned from tanks prior to the 07:30 feed. On-grown prawns were graded into similar weight classes, and stocked randomly into each tank of the experimental system. Four tanks per day were stocked with 2 prawns each to achieve a staggered start; to ensure that the prawns were fed feeds for similar durations at the time of staggered final sampling. Prawns were acclimated to the experimental system for 7 days where they were fed on a basal feed. No mortalities were observed during the 7 day acclimation period.

4.3.3 Feed formulation

The basal feed (control) was formulated at 47% crude protein, 7.3% crude lipid and 16.2 MJ.kg^{-1} of energy (Table 4.1) and contained similar ingredients to commercially available feeds (i.e. Ridley Aqua-Feed, Prawn Starter Enhance). The fucoidan feed (FV90) was formulated to contain 1.08 g.kg^{-1} fucoidan (*Fucus vesiculosus*, Marinova Pty Ltd, Cambridge, Tasmania) to provide a fucoidan concentration of 1 g.kg^{-1}, while α-cellulose (Sigma-Aldrich) was included in the control feed (basal) at 1.08 g.kg^{-1} to balance the formulation.

Fish meal (Skretting Australia, Cambridge, Tasmania, Australia) and krill meal (Ridley Aqua-Feeds, Narangba, Queensland, Australia) were included as sources of protein and lipid, while krill meal was also a feed attractant. Wheat flour (Four Roses, New South Wales, Australia) and wheat gluten (MP Biomedicals, Santa Ana, CA, USA) were binders. Cholesterol (Sigma-Aldrich, St. Louis, MO, USA) was added at 1 g.kg^{-1} and soy lecithin (Lucas Meyer Eurologistic, IIIinois, USA) at 10 g.kg^{-1}. Macro mineral premix was made from individual minerals (Sigma-Aldrich, St. Louis, MO, USA) and were included at 33 g.kg^{-1}, containing the recommended mineral concentrations for marine shrimp (Davis and Lawrence, 1997). Vitamin and micro mineral premix was made from individual vitamins and minerals (Sigma-Aldrich St. Louis, MO, USA) and was added at 1 g.kg^{-1} according to the recommended vitamin and mineral concentrations for marine shrimp (Conklin, 1997; Davis and Lawrence 1997) (Table 3.1).

Fish meal was sifted through a 1 mm sieve (Greer and Ashburner, 1-C). Dry bulk ingredients were homogenised in a mixer (Brice Australia, Pty Ltd, VFM-20C) for 20 min. Ingredients formulated in small inclusions, including fucoidan and α-cellulose were homogenised in a subsample of the bulk mix, then returned to the bulk mix for further mixing (20 min). Lecithin and choline chloride (MP Biomedicals) was dissolved in distilled water and added to

the dry mixture until crumbly dough could be formed. The dough was then passed twice through a 2 mm die of a commercial pasta maker (ItalPast, Mac 10s) and cut to approximately 4 mm lengths. Pellets were steamed for 10 min (Tefal, Steam Cuisine) and air dried (Forma Scientific, 68732-1) until feeds contained less than 10% moisture. Moisture content of feeds was determined by drying 2 g samples for 2 h at 135°C. Feeds were stored in airtight bags at -20°C until required.

Table 4.1. Ingredient inclusion 'as is' ($g.kg^{-1}$) and mean chemical composition (n=2) of experimental feeds ($g.kg^{-1}$ DM) ± S.E.

	Feeds	
Ingredients (as is)	**BASAL**	**FV90**
Fishmeal	500.00	500.00
Wheat flour	321.62	321.62
Krill meal	60.00	60.00
Wheat gluten	70.00	70.00
Fucoidan (FV90)[1]	0.00	1.08
α-cellulose	1.08	0.00
Macro mineral mix[2]	33.00	33.00
Lecithin	10.00	10.00
Vitamins & micro mineral mix[3]	1.00	1.00
Cholesterol	1.00	1.00
Stay C	1.00	1.00
Pigment	0.50	0.50
Banox E	0.20	0.20
Choline chloride	0.60	0.60
Chemical composition $g.kg^{-1}$		
Dry matter	884.91 ± 0.80	886.10 ± 1.32
Crude protein	525.77 ± 0.02	525.71 ± 0.05
Crude lipid	106.74 ± 2.17	106.64 ± 0.21
Gross energy ($MJ.kg^{-1}$)	19.25 ± 0.03	19.13 ± 0.02
Ash	60.48 ± 0.56	60.58 ± 0.10
Protein:energy ($g\ CP.MJ\ GE^{-1}$)	27.31	27.48
Fucoidan (%w/w)	0	0.14

Feeds contain: BASAL = Control, FV90 = Maritech® *Fucus vesiculosus*.
[1]Maritech® (Marinova Pty Ltd, Cambridge, Tasmania, Australia).
[2]Macro mineral mix (Davis and Lawrence, 1997), (g.kg): K_2PO_4, 10; $Ca(H_2PO_4)_2$, 10; NaH2PO4, 10; $MgSO_4.7H_2O$, 0.3.
[3]Vitamin and micro mineral premix (Conklin, 1997; Davis and Lawrence, 1997). Vitamins (mg.kg): thiamine-HCl, 60; riboflavin, 25; nicotinic acid, 40; pyridoxine-HCl, 50; pantothenic acid, 75; biotin, 1; folic acid, 10; vitamin B12, 0.2; myo-inositol, 400; L-ascorbic acid, 200; vitamin A acetate, 10; DL-α-tocopherol acetate, 100; vitamin D3, 0.1; menadone sodium bisulfite, 5, α-cellulose, 23.7. Micro minerals (mg.kg): $CuSO_4.5H_2O$, 32; $ZnSO_4.7H_2O$, 15; Na_2SeO_3, 0.2; α-cellulose, 52.

4.3.4 Juvenile prawns - Acute respiratory response to stress

Juvenile prawns (PL 71) were individually weighed (mean weight 0.88 ± 0.02 g) and feed treatments assigned in blocks of two tanks. There were 24 replicate prawns per feed treatment. The feeding period aimed to conclude at 21 d, however the actual number of days prawns fed feeds ranged from 16 to 25 days (mean number of days = 20.1 ± 0.52 days) due to standardisation of moult staging for respirometry measurements. Prawns were fasted for 24 h to exclude the influence of specific dynamic action and moult staged through observation of cuticle development of the uropods (Smith and Dall, 1985). Only intermoult (stage C) prawns were selected for measurement and were weighed before stress exposure for calculating $\dot{M}o_2$.

Immediately following feed trials one prawn from feed treatment (basal and FV90) was subjected to a constant decrease in water temperature (1°C h^{-1}) and salinity (3‰ h^{-1}) until 24°C and 20‰ salinity were achieved (de la Vega et al., 2006; Bett and Vinatea, 2009) (Table 4.2). Prawns were placed into 3 L (15.5 x 14 x 15 cm) mesh aquarium separation cages (Aqua One) which were moved between 25 L static tanks at each time period, which tank contained the predetermined water temperature and salinity parameters. Time 0 was indicative of the water temperature and salinity of the initial culture system (experimental conditions), while Time 7 is representative of stressful water parameters within the respirometry system. Immediately after stress exposure protocol the $\dot{M}o_2$ of prawns was measured under low temperature (24°C) and salinity (20‰) conditions within the respirometry system as outlined in section 4.3.6.

4.3.5 Sub-adult prawns – Chronic respiratory response to stress

Sub-adult prawns were individually weighed (mean weight 19.08 ± 0.62 g) and feed treatments assigned in blocks of two tanks. There were 24 prawns fed the basal feed treatment and 23 prawns fed the FV90 feed treatment. The feeding period aimed to conclude at 21 d, however the actual number of days prawns fed feeds ranged from 17 to 36 days (mean number of days = 26.0 ± 0.61 days). Larger prawns were used in this experiment and times between moulting were extended due to the longer intermoult duration. The feeding regime was extended until one prawn from each feed treatment was in the correct moult stage. Prawns were weighed, moult staged (stage C, D0 and D1) before exposure to stress or non-stress conditions. Prawns were then exposed to the stress or non-stress conditions as per the protocol (Table 4.3) before being allocated a further 72 h to acclimate to the treatment exposure. During this period, prawns were maintained in a 118 L static culture tank under non-stress (30°C and 40‰ salinity) or stress conditions (24°C and 20‰ salinity) at reduced light conditions (24 h light cycle). Water temperature during this period was maintained by aquarium heaters (Aqua One, 60 W) and air-stones provided continuous aeration. During this period, prawns were not fed and a 20% water change was conducted daily, to reduce the build-up of toxic nitrogenous waste. Prawns in stage C, D0 and D1 were included in statistical analyses due to prawns advancing into early stages of pre-moult during this 72 h recovery period and prawns in these stages would usually be feeding.

Immediately after the 72 h acclimation period the $\dot{M}O_2$ of one prawn from each feed treatment was measured under low temperature (24°C) and salinity (20‰) conditions for the stress treatment or under optimal condition (30°C and 40‰) for the non-stress treatment, within the respirometry system as outlined in section 4.3.6.

Table 4.2. Acclimation protocols for juvenile *Penaeus monodon* held under stress (24°C & 20‰) conditions, prior to measuring $\dot{M}o_2$.

Juvenile		
Stress group		
Time (h)	Temp (°C)	Salinity (‰)
0	30	40
1	29	37
2	28	34
3	27	31
4	26	28
5	25	25
6	24	22
7	24	20

BASAL and FV90 (n = 24 per feed treatment)

Table 4.3. Acclimation protocols for sub-adult *Penaeus monodon* held under stress (24°C & 20‰) and non-stress control (30°C & 40‰) conditions, prior to measuring $\dot{M}o_2$.

Sub-adult					
Control group			Stress group		
Time (h)	Temp (°C)	Salinity (‰)	Time (h)	Temp (°C)	Salinity (‰)
0	30	40	0	30	40
1	30	40	1	29	37
2	30	40	2	28	34
3	30	40	3	27	31
4	30	40	4	26	28
5	30	40	5	25	25
6	30	40	6	24	22
7	30	40	7	24	20

BASAL non-stress control (n = 11)
FV90 non-stress control (n = 10)
BASAL stress (n = 13)
FV90 stress (n = 13)

4.3.6 Oxygen consumption rate measurement

Metabolic ($\dot{M}o_2$) rates were measured using a two chamber automated intermittent-flow static respirometry system, previously described by Jensen et al. (2013) (Figure 4.1). The system components were immersed in a 160 L white high density polypropylene tank (60 x 60 x 46 cm). Water was treated by a UV sterilizer (UV Guard Australia Pty Ltd, 40 W) to reduce microbial proliferation, which can impact on DO rates during measurements. For stress conditions (juvenile and sub-adult prawns) water temperature was maintained at 24°C by a 2 kW digitally controlled heater (Instra) and salinity made to 20‰ using de-chlorinated tap water and a salinity refractometer (IWAKI, TD5Q8). For non-stress conditions (sub-adult prawns control group), prawns in the control group (30°C, 40‰) were maintained at 30°C and salinity was made to 40‰ using pool salt (Cheetham Salt, Mermaid Finest, Australia) and was measured using a salinity refractometer (IWAKI, TD5Q8).

Air-tight respirometry chambers were constructed from a plastic sample container with screw-on lids, secured horizontally to a submerged frame. Two hundred and fifty ml chambers were used for juvenile prawns, and either 500 or 1000 ml chambers for sub-adult prawns, due the lager mass of individuals. Plastic oyster mesh (4 mm^2) substrates were placed in each container to provide a substrate for prawns to grip and to remain in an upright orientation to reducing stress and movement during O_2 measurements (Dall, 1986). Respirometry chambers were plumbed using low permeability tubing (Tygon® R-3603) and secured with lure lock fittings to further reduce O_2 permeability between chambers and sump water. During all respiratory measurements, prawns were subjected to a 24 h light photoperiod low light conditions. The 24 h light regime aimed to reduce diurnal rhythms in O_2 consumption rates, normally experienced between light and dark cycles.

Dissolved oxygen concentrations for each chamber were measured and logged every minute using two luminescent dissolved oxygen probes (HACH, HQ40d) housed within 14.5 ml Perspex chambers and sealed air tight. Water was continuously recirculated through respirometry and DO probe chambers (closed cycle) by individual submersible pumps (Aqua One, Quiet One 1200). A second pump introduced new water (open cycle) from the sump to the chambers every 20 min for 10 min duration (juvenile prawns); and every 10 min for 5 min duration for sub-adult prawns, controlled by an automatic recycling timer (Sentinel, DRT-1). During the closed cycle a constant drop in oxygen concentration ($mg.L^{-1}$) was observed until the open cycle was activated, replenishing DO concentrations in chambers. Oxygen consumption rates of prawns were determined by applying linear regressions to the rate of decline of DO concentration over the final 9 min of each 10 min respirometer closed cycle period (juvenile prawns); and final 4 min of each 5 min closed cycle for sub-adult prawns. On occasion when linear regression coefficients were $<R^2 = 0.70$, data for that period were excluded from analysis; accounting for 1.67 and 1.50% of measurements (juvenile and sub-adult prawns respectively). During the closed cycle, oxygen saturation within the chambers did not drop below 70% oxygen saturation for juvenile prawns. However, for sub-adult prawns, the oxygen saturation dropped below 70% on six occasions (lowest = 49.2% O_2) during recordings under non-stress conditions. All respirometry data were adjusted for the chamber volumes.

At the start of the respirometry experiment prawns were placed in respirometry chambers at approximately 16:00 where they remained until approximately 08:00 the next day or 16 h after stocking the chambers. Data collected during this time (16 h) were used to calculate the standard metabolic (SMR) and routine metabolic rates (RMR). Prawns were removed one at a time during the open cycle and transferred to a 25 L black polypropylene tank, where they were chased by hand until exhaustion (Fitzgibbon et al., 2014). Prawns were transferred back

to the chamber approximately 10 seconds prior to the start of the next closed cycle and DO recorded for 4 h to measure the active metabolic rate (AMR). Prawns were removed and the chamber DO was recorded for an additional one hour to determine background O_2 consumption, due to the system components and microbial growth. Between respiratory trials sodium hypochlorite was added to the system at 10 mg/L for 1 h to disinfect respiratory components and rinsed with freshwater prior to refilling and starting another trial.

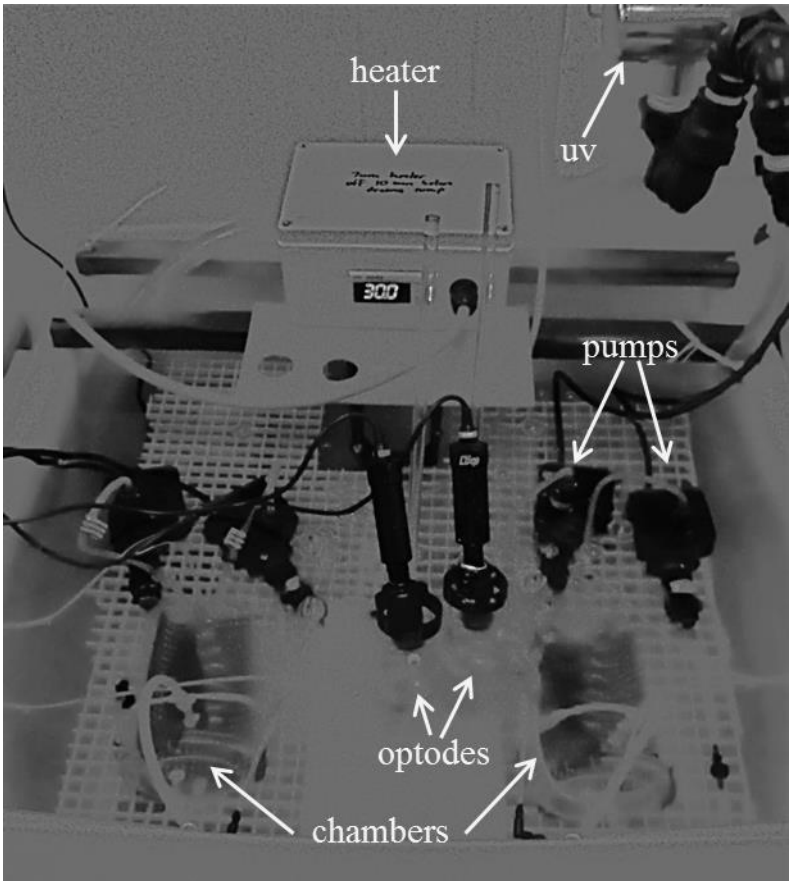

Figure 4.1. Respirometry system showing chambers, submersible pumps, dissolved oxygen optodes, heater and ultraviolet sterilizer.

4.3.7 Viral and immune response sampling

At the conclusion of the respirometry measurements for sub-adult, all prawns were weighed and moult staged a second time prior to removing haemolymph for biochemical analyses. Approximately 100 µl of haemolymph was removed from the cardiac sinus using a 25 gauge needle and 1 ml tuberculin syringe, rinsed with chilled Shrimp Salt Solution (SSS) (450 mM NaCl, 10 mM KCl, 10 mM EDTA.Na_2, 10 mM HEPES, pH 7.3, 850 mOsm kg^{-1}) (Vargas-Albores et al., 1993) and immediately ejected into a 2 ml microcentrifuge tube. Three aliquots of the haemolymph were quickly added to individual tubes: 50 µl haemolymph in 200 µl SSS for phenoloxidase activity (PO activity), 25 µl haemolymph in 25 µl formalin for total haemocyte counts (THC) and 25 µl haemolymph in 25 µl formalin for granular haemocyte counts (GHC). For GAV analysis pleopods were dissected at the base of the exopod and quickly transferred to a nucleic acid preservation solution (NAPS, 4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5) as described by Polinski et al. (2013). Pleopod samples were kept at 4°C for 24 h prior to storing at -20°C for gill-associated virus (GAV) analysis.

Viral analysis was conducted at the CSIRO Animal, Food & Health Sciences, Queensland Bioscience Precinct, Queensland. The analysis was conducted as described by de la Vega et al. (2004). Briefly, pleopods were homogenised in TRIzol® Reagent (Life Technologies) using standard manufacturers protocol to extract RNA. The RNA was resuspended in 35 µl RNase/DNase-free water. The RNA was used for the synthesis of cDNA (Tetro cDNA synthesis kit, BIOLINE Cat no. 65043) using random primers as per manufacturer's protocol.

4.3.8. Chemical analyses

Sub-samples of experimental feeds were freeze-dried (Dynavac, FD3) to a constant weight, and chemical composition analysed in triplicate. Feeds were homogenised to a fine powder using a bench-top hammer mill (Culatti micro hammer-cutter mill, MFC). Samples were wrapped in aluminium foil and placed in an airtight plastic bag at -20°C until required.

Crude protein analysis was conducted using Kjeldahl method (FOSS Kjeltec™ 8100), crude protein was calculated as N x 6.25, crude lipid (Bligh and Dyer, 1959), gross energy by bomb calorimetry (Gallenkamp Autobomb, CAB101), ash by combustion (SEM 102C muffle furnace) at 600°C for 2 h, using standard laboratory methods in accordance with AOAC (1995). The moisture content of feeds was determined by drying a 2 g sample of feed in triplicate at 135°C to a constant weight.

All analyses for the determination of fucoidan, polyphenol and sulfate concentrations were conducted by determining fucose concentration in freeze-dried prawn feeds and back-calculating to determine final fucoidan concentration in feeds. Total carbohydrate content was determined by spectrophotometric analysis of the hydrolysed compound in the presence of phenol, a proprietary method of Marinova Pty Ltd based on Dubois et al. (1956). Sulfate content was analysed spectrophotometrically using $BaSO_4$ precipitation method ($BaCl_2$ in gelatin), based on Dodgson (1961) and Dodgson and Price (1962). Analysis of polyphenol content was also determined spectrophotometrically using modified method based on the Folin-Ciocalteu reagent (phosphomolybdate/phosphotungstate) (Zoecklein et al., 1999; Jimenez-Escrig et al., 2001; Zhang et al., 2006). The carbohydrate profile was determined using a GC-based method for the accurate determination of individual monosaccharide ratios, using acetylated alditol derivatives in the hydrolysed samples (Morvai-Vitanyi et al., 1993). Molecular weight profiles were determined by gel permeation chromatography, with the aid

of a size exclusion column, reported relative to Dextran standards. Due to the low inclusion of fucoidan in feeds (0.1% inclusion) the fucoidan concentration was determined by multiplying the fucose concentration by the fucose content by a known species and process factor, derived from the initial source fucoidan product.

4.3.9 Immune response

4.3.9.1 Total haemocyte count (THC)

One drop (20 µl) of formalin fixed haemolymph mixture was placed on a Neubauer Improved Bright-line haemocytometer under 400x magnification of a light microscope (Olympus, CH30). At least 200 haemocytes were counted in triplicate for quantifying the total number of haemocytes per ml^{-1} of haemolymph.

4.3.9.2 Granular haemocyte count (GHC)

Formalin fixed haemolymph was used for GHC according to Sritunyalucksana et al. (2005). After fixing haemocytes in formalin for approximately 20 min, 50 µl of Rose Bengal solution (Sigma-Aldrich, 1.2% Rose Bengal in 50% ethanol) was added to the micro-centrifuge tube and incubated at room temperature for 20 min (Sritunyalucksana et al., 2005). Stained haemolymph smears from individual prawns were made using one drop (20 µl) of stained haemolymph solution smeared onto a clean microscope slide. Smears were air-dried, then counterstained with haematoxylin (Sigma-Aldrich) for 3 min, before rinsing in tap water for 2 min followed by immersion for 1 min in 95% iso-propyl alcohol and 2 min in 100% iso-propyl alcohol (repeated twice), using an automated diversified stainer (Sakura, DRS-60) (Sritunyalucksana et al., 2005). Following dehydration, slides were placed in xylene for 2 min and mounted with a cover-slip using DPX mountant (Sigma-Aldrich). Granulocyte ratios (DHC) were determined by counting 200 haemocytes per smear under 400 x objective of a

light microscope (Olympus BH-2) and expressed as a proportion of granulocytes (GHC) (small-granular and large-granular haemocytes) in 200 total haemocytes, i.e. count/200 x THC (Sritunyalucksana et al., 2005).

4.3.9.3 Phenoloxidase (PO) activity

The PO activity method followed the protocol from Hernandez-Lopez et al. (1996) using the modified 96 well micro-plate method. Haemolymph diluted in SSS (1 in 4 dilution) was centrifuged (Eppendorf, S415D) at 300 x *g* at 4°C for 5 min and the supernatant was removed. The cell pellet was resuspended with 200 µl cacodylate buffer (10 mM sodium cacodylate, 10 mM $CaCl_2$, pH 7.0).

PO activity was measured spectrophotometrically (in triplicate) by the formation of dopachrome from the substrate, L-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich). Fifty microliters of pooled haemocyte cell suspension was incubated with 50 µl of laminarin (Sigma-Aldrich) (1 mg/ml in cacodylate buffer) in a flat-bottomed 96 well micro-plate (TTP®, 92696) for 30 min at 25°C. Fifty microliters of L-DOPA (3 mg/ml in cacodylate buffer) was added to each well and incubated at 25°C for 10 min, before reading the optical density at 492 nm on an ELISA reader (SPECTRA, Rainbow Thermo). Enzyme activity was expressed as the change in absorbance per min (Δ Abs.min^{-1}) per 100 µl of haemolymph.

4.4 Calculations

Routine metabolic rate (RMR mg gWW^{-1} h^{-1}) was calculated as:

$RMR = \dot{M}o_2 / W_{wet}$

$\dot{M}o_2$ = drop in O_2 (mg/h) during closed cycle x respirometry chamber volume (L) – the average background O_2 consumption over 1 h in chamber minus prawn; W_{wet} = the wet weight of the prawn (g).

Standard metabolic rate (SMR mg gWW^{-1} h^{-1}) was calculated as:

SMR = Mean of 5 times lowest RMR

Where: Mean of the five lowest measures of RMR recorded over 16 h.

Maximal metabolic rate (MMR mg gWW^{-1} h^{-1}) was calculated as:

MMR = Mean of 5 times highest RMR

Where: Mean of the five highest measures of RMR recorded over 16 h.

Active metabolic rate (AMR mg gWW^{-1} h^{-1}) was calculated as:

AMR = largest $\dot{M}o_2$ recorded

Where: the largest $\dot{M}o_2$ concentration value recorded after chasing prawn to exhaustion.

Aerobic scope (A-SCOPE mg gWW^{-1} h^{-1}) was calculated as:

A-SCOPE = AMR – SMR

Where: AMR is the largest $\dot{M}o_2$ concentration value recorded after chasing prawn to exhaustion; SMR is the mean of the five lowest measures of recorded over 16 h.

Total haemocyte count (THC cells.ml^{-1}) was calculated as:

$THC = (MHC \times 2.5 \times 10^5) \times DF$

Where: MHC = mean no. of haemocytes per medium square of the haemocytometer; 2.5×10^5 = conversion factor, changing millimetres to millilitres; DF = dilution factor, cell concentration diluted by addition of formalin fixative and Rose Bengal stain (Sritunyalucksana et al., 2005).

Granular haemocyte count (GHC cells.ml^{-1}) was calculated as:

GHC = (GH x THC)

Where: GH = total number of small and large granular haemocytes; THC = mean number of haemocytes per ml of haemolymph (Sritunyalucksana et al., 2005).

4.5 Statistical analyses

For the feeding trial, each tank was an individual replicate unit for calculating mean survival. Each prawn in the respirometry chamber of the respirometry unit was considered a single replicate unit. Test of equal variances was assessed using Levene's test of equality and residual plots. For juvenile prawns, means were compared using an Independent sample T-Test for comparisons between metabolic parameters, results of one-tailed tests were considered significant at $P < 0.05$. Survival percentage data were arcsine transformed and data containing zero values were arcsine transformed by the square root of the survival as a proportion. For sub-adult prawns, interactions between treatment and stressor for immune responses, and $\dot{M}o_2$ parameters were compared by separate two-way Analysis of Variance (ANOVA). Results were considered significant if $P < 0.05$ using a Tukey's HSD test. Factors which were non-significant and where there was no significant interaction ($P > 0.05$) were pooled by stressor and analysed by one-way ANOVA. Data of unequal variances were square root transformed before running a statistical test. Statistical analysis was conducted using IBM SPSS STATISTICS (VERSION 21). Tables and figures were constructed using Microsoft Office Excel 2010 and Sigma Plot, version 11.0 (Systat Software, Inc).

4.6 Results

4.6.1 Survival (Juvenile prawns)

There were no significant differences in mean survival rates of juvenile prawns fed the basal (50 ± 12.91%) and FV90 (60 ± 10.00%) treatments respectively (t = -0.518, df = 22, P = 0.610) (Figure 4.2).

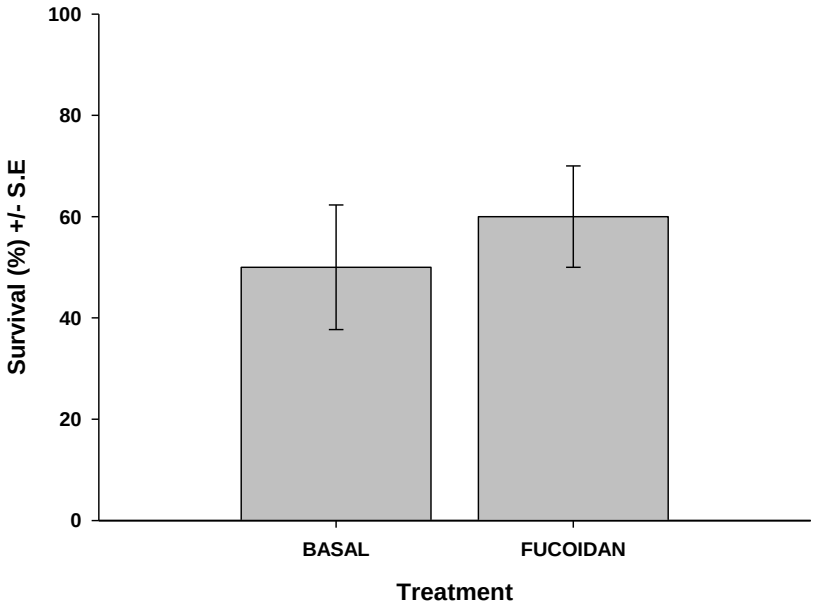

Figure 4.2. Mean survival (%) ± S.E. of juvenile *Penaeus monodon* fed basal (control) and fucoidan (FV90) feeds (t = -0.518, df = 22, P = 0.610). n = 24 per feed treatment. $P < 0.05$ was considered significant.

4.6.2 Aerobic metabolic rate (Juvenile prawns)

There were no significant differences in SMR (t = -0.321, df = 16, P = 0.376), RMR (t = -0.329, df = 16, P = 0.374) and MMR (t = -0.072, df = 16, P = 0.472) between juvenile prawns fed the basal and FV90 feed treatments (Figure 4.3). Juvenile prawns fed the FV90 treatment had a significantly higher mean AMR (t = -1.914, df = 16, P = 0.037) than prawns fed the basal treatment (Figure 4.3). There were no significant differences in the aerobic scope of prawns fed both treatments using a one-tailed test (t = -1.601, df = 16, P = 0.064) (Figure 4.3).

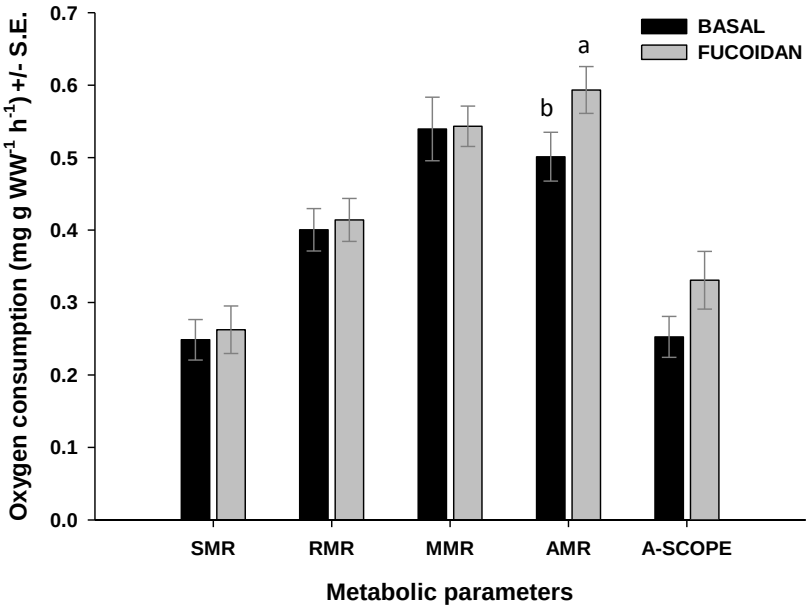

Figure 4.3. Mean metabolic oxygen consumption (mg gWW^{-1} h^{-1}) rates (± S.E.) of juvenile *Penaeus monodon* fed basal (control) and FV90 (fucoidan) treatments prior to measurements of standard metabolic rate (SMR), routine metabolic rate (RMR), maximal metabolic rate (MMR), active metabolic rate (AMR) and (A-SCOPE) aerobic scope measured over 20 h. Means with different superscripts are significantly different (P < 0.05 was considered significant). n = 9 per feed treatment.

4.6.3 Gill associated virus concentration (Juvenile prawns)

All juvenile prawns in each feed treatment tested positive for GAV and there was no significant difference in the viral concentration of prawns fed both treatments (F = 0.225, df = 1, 16, *P* = 0.642) (Figure 4.4). Mean GAV concentration of *P. monodon* fed the basal treatment was 0.93 ± 0.13 and for prawns fed the FV90 the mean was 0.98 ± 0.24 GAV copies/ng RNA.

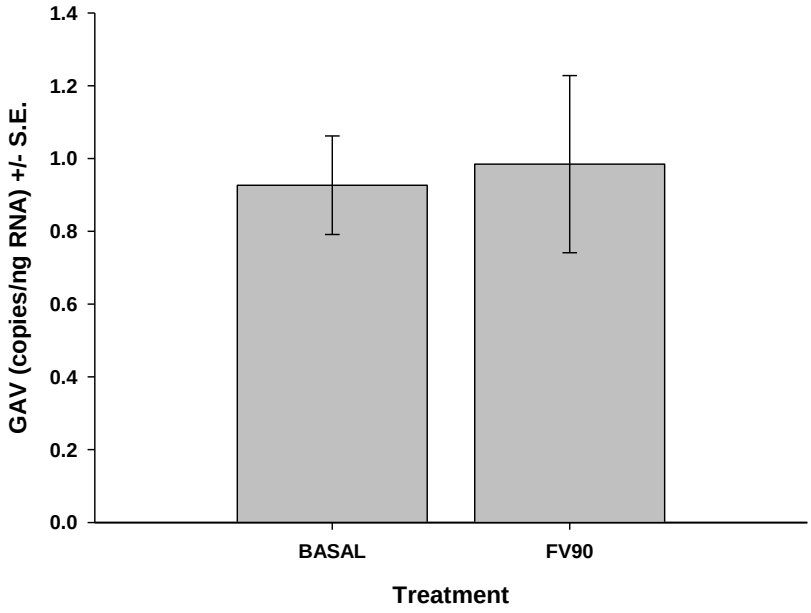

Figure 4.4. Mean GAV (Gill Associated Virus) (copies/ng RNA ± S.E.) of juvenile *Penaeus monodon* fed basal (control) and FV90 (fucoidan) after stress treatment (F = 0.225, df = 1, 16, *P* = 0.642). $P < 0.05$ was considered significant. n = 9 per treatment.

4.6.4 Survival (Sub-adult prawns)

There were no significant differences in mean survival rates of sub-adult prawns, survival of prawns fed the basal and FV90 treatments were 96.88 ± 3.12% and 100% respectively at the conclusion of the feeding trial (F = 0.902, df = 1, 57, *P* = 0.346) (Figure 4.5).

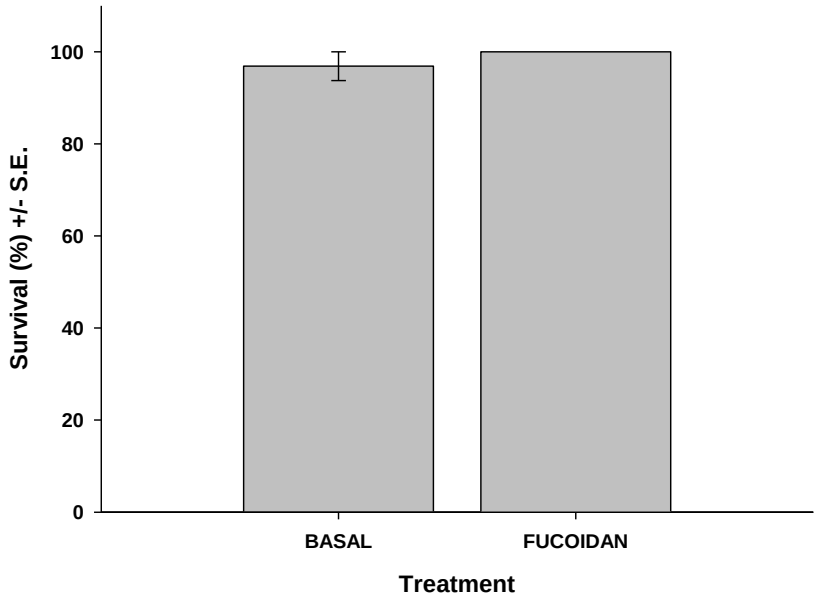

Figure 4.5. Mean survival (%) ± S.E. of sub-adult *Penaeus monodon* after feeding on basal (control) and FV90 (fucoidan) feeds (F = 0.902, df = 1, 57, *P* = 0.346). $P < 0.05$ was considered significant. n = 26 per feed treatment.

4.6.5 Aerobic metabolic rate (Sub-adult prawns)

When sub-adult prawns were fed the basal and FV90 treatments there were no significant interactions ($P > 0.05$) between feed treatments and stressor treatments for all measured metabolic oxygen consumption parameters SMR, RMR, MMR, AMR and A-SCOPE (Table 4.4). Therefore, data from feed treatments were pooled to assess the effect of stress. After pooling the oxygen consumption (SMR, RMR, MMR, AMR and A-SCOPE) of prawns under non-stress conditions were significantly higher ($P < 0.001$) than prawns under stressful conditions (Figure 4.6).

4.6.6 Immune response (Sub-adult prawns)

There were no significant interactions ($P > 0.05$) of feed and stressor treatments on the measured immune response (THC, GHC and PO activity) in sub-adult *P. monodon* (Table 4.5). When data were pooled for different feed treatments, THC, GHC and PO activity prawns kept under stress conditions had significantly higher ($P < 0.05$) responses (Figures 4.7 to 4.9). On average prawns held under stress conditions had 25% more total haemocytes and 39% more granular haemocytes respectively than prawns under optimal conditions (Figures 4.7 and 4.8). The PO activity of prawns held under stress conditions had on average 61.7% higher PO activity than prawns under optimal conditions (Figure 4.9).

Table 4.4. Mean metabolic oxygen consumption (mg gWW^{-1} h^{-1}) rates (± S.E.) of sub-adult *Penaeus monodon* maintained under non-stress and stress conditions and fed basal (control) and FV90 (fucoidan) treatments.

Metabolic rates	**Non-stress**		**Stress**		**Two-way ANOVA**		
($\dot{M}O_2$)	**BASAL**	**FV90**	**BASAL**	**FV90**	**F-value**	**df**	***P***
SMR	0.20 ± 0.01	0.20 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	1.563	1, 42	0.218
RMR	0.36 ± 0.02	0.36 ± 0.01	0.27 ± 0.02	0.26 ± 0.01	0.107	1, 43	0.746
MMR	0.60 ± 0.03	0.58 ± 0.02	0.45 ± 0.03	0.43 ± 0.02	0.017	1, 43	0.897
AMR	0.71 ± 0.04	0.75 ± 0.03	0.49 ± 0.02	0.51 ± 0.01	0.254	1, 43	0.617
A-SCOPE	0.51 ± 0.03	0.55 ± 0.09	0.37 ± 0.02	0.40 ± 0.01	0.021	1, 43	0.885

Feeds contain: BASAL = Control, FV90 = Maritech® *F. vesiculosus*.
Metabolic rates (mg g WW^{-1} h^{-1}): SMR = standard metabolic rate, RMR= routine metabolic rate, MMR = maximal metabolic rate, AMR = active metabolic rate, A-SCOPE = aerobic scope.
SMR: BASAL & FV90 Non-stress (n = 11, n = 10), BASAL & FV90 Stress (n = 12, n = 13).
RMR: BASAL & FV90 Non-stress (n = 11, n = 10), BASAL & FV90 Stress (n = 13, n = 13).
MMR: BASAL & FV90 Non-stress (n = 11, n = 10), BASAL & FV90 Stress (n = 13, n = 13).
AMR: BASAL & FV90 Non-stress (n = 11, n = 10), BASAL & FV90 Stress (n = 13, n = 13).
A-SCOPE: BASAL & FV90 Non-stress (n = 11, n = 10), BASAL & FV90 Stress (n = 13, n = 13).
$P < 0.05$ was considered significant.

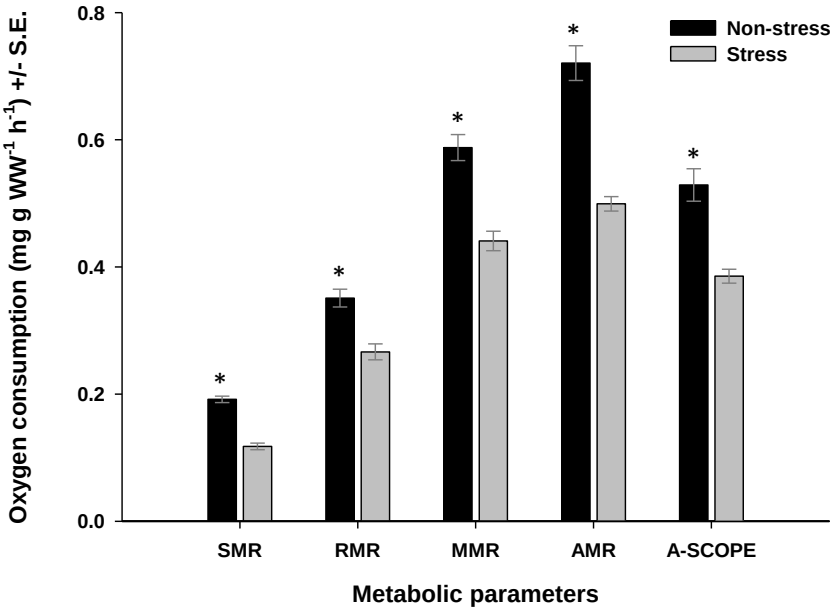

Figure 4.6. Mean oxygen consumption rates (mg gWW^{-1} h^{-1}) of sub-adult *Penaeus monodon* (± S.E.), pooled by stressor for standard metabolic rate (SMR), routine metabolic rate (RMR), maximal metabolic rate (MMR), active metabolic rate (AMR) and (A-SCOPE) aerobic scope measured over 20 h. Means with asterisk are significantly different for each metabolic parameter (P = <0.05). $P < 0.05$ was considered significant.

Table 4.5. Mean immune response variables (± S.E.) of sub-adult *Penaeus monodon* maintained under non-stress and stress conditions within a respirometry chamber and fed basal (control) and FV90 (fucoidan) treatments.

		Non-stress		Stress		Two-way ANOVA		
Immune response	**Units**	**BASAL**	**FV90**	**BASAL**	**FV90**	**F-value**	**df**	***P***
THC	cells.ml^{-1}x 10^{7}	1.99 ± 0.36	2.13 ± 0.19	2.93 ± 0.26	2.55 ± 0.16	0.824	1, 36	0.370
GHC	cells.ml^{-1} x 10^{7}	0.76 ± 0.15	1.45 ± 0.17	0.87 ± 0.16	1.24 ± 0.10	0.982	1, 35	0.329
PO activity	Δ Abs.min^{-1}	0.027 ± 0.01	0.031 ± 0.01	0.080 ± 0.01	0.088 ± 0.01	0.015	1, 44	0.905

Feeds contain: BASAL = Control, FV90 = Maritech® *F. vesiculosus*.
Immune responses: THC = total haemocyte count, GHC = granular haemocyte count, PO activity = phenoloxidase activity.
THC: BASAL & FV90 Non-stress (n = 7, n = 5), BASAL & FV90 Stress (n = 14, n = 14).
GHC: BASAL & FV90 Non-stress (n = 7, n = 5), BASAL & FV90 Stress (n = 13, n = 14).
PO activity: BASAL & FV90 Non-stress (n = 9, n = 10), BASAL & FV90 Stress (n = 15, n = 14).
$P < 0.05$ was considered significant.

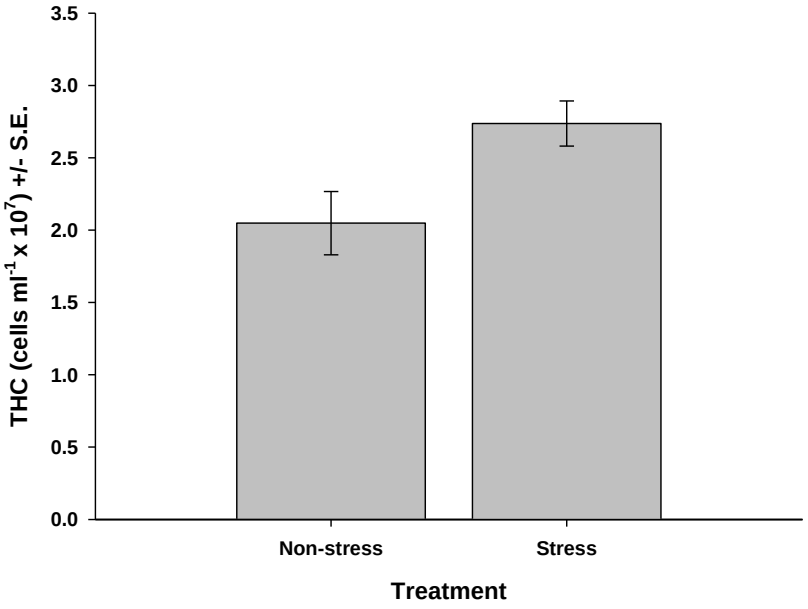

Figure 4.7. Mean total haemocyte counts (THC) (cells ml^{-1} x 10^7 ± S.E.) of sub-adult *Penaeus monodon* pooled by stress and non-stress treatments (F = 5.854, df = 1, 38, $P = 0.020$). $P < 0.05$ was considered significant.

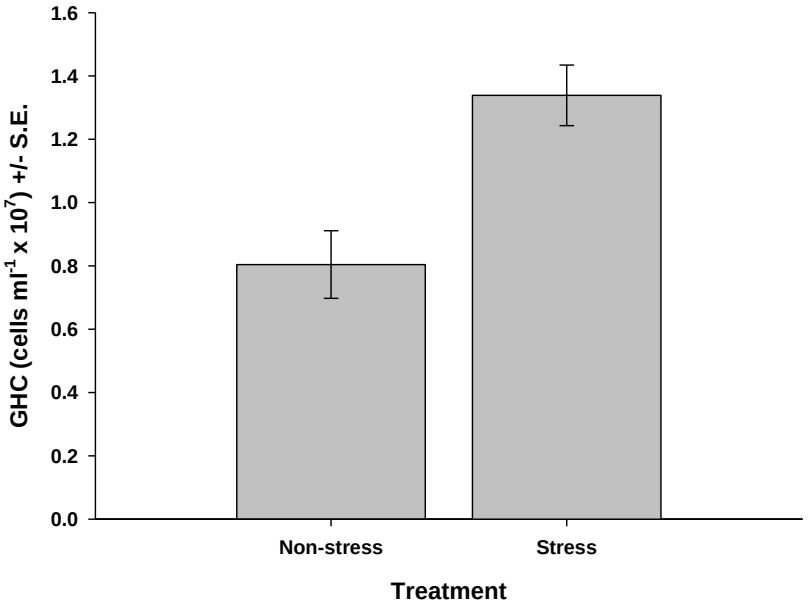

Figure 4.8. Mean granular haemocyte counts (GHC) (cells ml^{-1} x 10^7 ± S.E.) of sub-adult *Penaeus monodon* pooled by non-stress and stress treatments (F = 9.052, df = 1, 37, $P = 0.005$). $P < 0.05$ was considered significant.

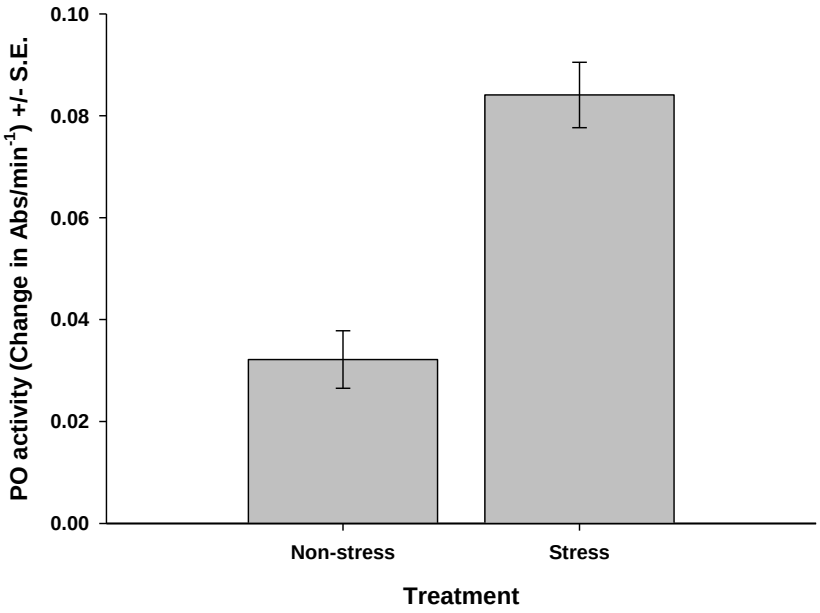

Figure 4.9. Mean phenoloxidase activity (PO activity) (Δ in Abs.min ± S.E.) of sub-adult *Penaeus monodon* pooled by stress and non-stress treatments (F = 32.133, df = 1, 46, P <0.001). $P < 0.05$ was considered significant.

4.6.7 Gill associated virus concentration (Sub-adult prawns)

All prawns in each feed treatment tested positive for GAV, however there was no significant interaction in the viral concentration of prawns fed both treatments held under stress or non-stress conditions (F = 0.638, df = 1, 51, *P* = 0.428) (Figure.4.10).

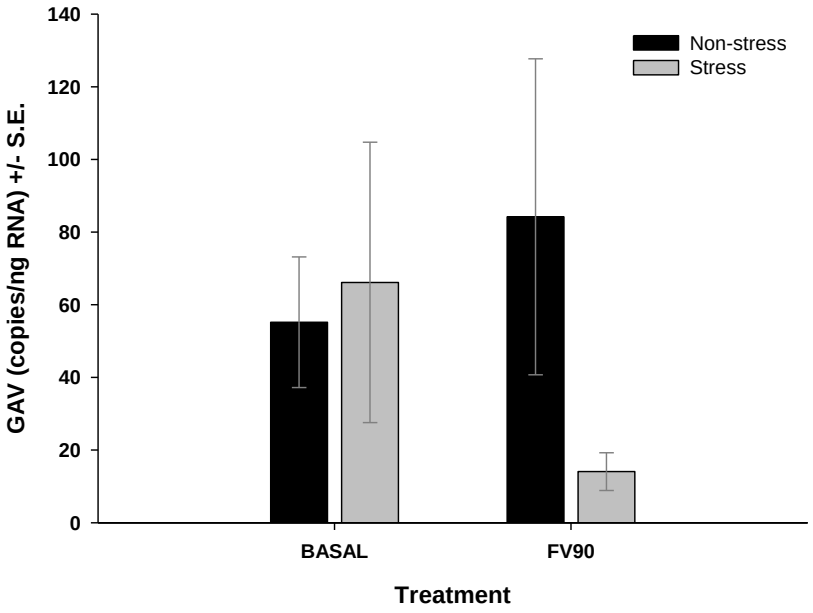

Figure 4.10. Mean GAV (Gill Associated Virus) (copies/ng RNA ± S.E.) of sub-adult *Penaeus monodon* by stress and non-stress treatments (F = 0.638, df = 1, 51, *P* = 0.428). $P < 0.05$ was considered significant.

4.7 Discussion

Two experiments were conducted with juvenile and sub-adult prawns to determine the effects of dietary fucoidan on aerobic capacity and immune response under acute (7 h) and chronic (72 h) stress conditions. Fucoidan failed to influence prawn survival. Prawns subjected to environmental stress had reduced aerobic capacity and exhibited a greater immune response in terms of THC, GHC and PO activity. Fucoidan had little effect on $\dot{M}o_2$, except for an increase AMR in juvenile prawns.

During the feeding trial for juvenile prawns mortality was high, although not different between prawns fed each feed treatment. All surviving prawns were sampled to obtain $\dot{M}o_2$ measurements and later 100% of prawns tested positive for GAV. GAV load, determined by RT-PCR was not different between feed treatments. In contrast to the results obtained from juvenile prawns under stressful conditions, the sub-adult prawns had very high survival rates for both FV90 (100%) and basal (96.7%) fed prawns, during the feeding trial. Sub-adult prawns had on average 80 times more GAV copies/ng RNA than that in the juvenile prawns. Low-level GAV of 0.04 to 338 GAV RNA copies/ng RNA were reported in *P. monodon* during a feeding study, and were determined to be pre-existing chronic infections, although growth performance and survival were not negatively affected (Sellars et al., 2015). In the current study the highest recorded GAV concentration was 120 GAV RNA copies/ng RNA, and was within the range of the GAV concentrations in the study by Sellars et al. (2015). It is commonly accepted that 100% of prawn broodstock from Queensland waters are chronically infected with GAV, and associated progeny spawned in hatcheries follow the same GAV prevalence (Cowley et al., 2000; Walker et al.,

2001). The prawn stock used in both trials was from the same cohort obtained from a Queensland hatchery, although sub-adult prawns were on-grown to a much larger body weight. An increased GAV concentration in larger prawns may be explained by the development of infection within cells, where larger prawns have an increased number of viral particles; however they are larger and therefore more resistant to the chronic infection as also observed under commercial farming conditions (Munro et al., 2011).

Handling and inoculation controls can elicit an increase in viral load from a chronic to an acute infection (de la Vega, 2004). In the study by de la Vega et al. (2004), initial GAV loads were approximately 400 ± 317 copies/ng of total RNA after inoculation with GAV, the viral load increased 10^5-fold by day 6, while even in saline injected control prawns, GAV load increased 10^3-fold. In the current research, juvenile prawns were repeatedly handled for determination of correct moult stage, moving mesh cages between decreasing temperature and salinity media and to obtain a wet weight. While sub-adult prawns were acclimated to the water parameters for 72 h, before being moult-staged and weighed a second time to ensure they had not progressed into a different stage prior to measuring $\dot{M}o_2$. The additional handling may explain why the sub-adult prawns had on average an 80-fold increase in GAV load and there were no unexplained mortality events during the feeding trial. In the current study, sub-adult prawns showed gross signs of stress, including reddening of uropods, pleopods and appendages (Perazzolo et al., 2002) observed at the conclusion of the 72 h acclimation period, prior to $\dot{M}o_2$ measurements.

The active metabolic rate of juvenile *P. monodon* was significantly higher when fed a feed containing fucoidan, under stressful conditions, while all other measured $\dot{M}o_2$ rates were the same, regarding whether prawns were fed fucoidan or not. Therefore, fucoidan can increase maximum aerobic capacity in juvenile prawns and thus potentially correspond to increased fitness, immune function or support oxygen-consuming functions such as muscular activity (Djawdan et al., 1997; Killen et al., 2007; Clark et al., 2013). However, this effect was not profound enough to significantly influence A-SCOPE. Possibly this is because all prawns in both treatments were infected with GAV. Disease has shown to cause down regulation of aerobic capacity in the Atlantic blue crab, resulting from initiating an immune response, or by actively regulating metabolic depression within the gills (Burnett et al., 2006; Thibodeaux et al., 2009).

There were no interactions of diet and stress or non-stress conditions on $\dot{M}o_2$ rates on sub-adult *P. monodon*. However, the pooled $\dot{M}o_2$ rates of prawns subject to stress conditions were significantly reduced when compared to prawns under non-stress conditions. This demonstrates that a combined drop in water temperature from 30°C to 24°C and salinity from 40‰ to 20‰ has negatively affected the aerobic capacity of sub-adult prawns. Similar results were determined when *Litopenaeus stylirostris* were exposed to a combined decrease in water temperature and salinity; the oxygen consumption of prawns was the lowest at 20°C and increased in direct proportion with temperature, more so than change in salinity within physiological limits (Spanopoulos-Hernandez et al., 2005). Physiological mechanisms such as the aerobic capacity of mitochondria are limited at low temperatures for tropical species such as *Penaeus monodon*; favouring anaerobic mitochondrial metabolism, decreasing available energy for maintenance (Portner 2002).

Sub-adult prawns held under non-stress conditions had a higher mean AMR; therefore, these prawns were able to consume more O_2, enabling increased energy for maintaining metabolic functions including immune function compared to prawns held under stressful conditions (Clark et al., 2013). Prior to sampling the immune responses, the non-stress sub-adult group were lethargic and showed gross signs of oxidative stress in the tail muscle, by the presence of opaque muscle tissue (Niu et al., 2013). The opaque muscle tissue could be due to the prawns exerting themselves prior, during chasing prior to recording final oxygen consumption readings. Therefore, the prawns held under non-stress conditions could be in fact "stressed" by physical stress, rather than environmental stress at least for the measures of immune response. It has been claimed that during periods of high energetic demands of evading predators or during environmental stress, there is less energy available for growth and increased energy is required to fulfil metabolic functions including maintenance for general homeostasis, such as in the current experiment where prawns were chased by hand (Villarreal et al., 2003; Killen et al., 2007).

Acute environmental fluctuations have shown to cause a decrease in the total number of haemocytes in prawns (Le Moullac and Haffner, 2000). In the current study, there was no interaction of diet on the THC and GHC of prawns at the conclusion of $\dot{M}o_2$ measurements. However prawns pooled by stressor treatment show that prawns held under stress conditions had more THC and GHC than those under non-stress conditions. There is much evidence suggesting that a stress event causes a rapid decline in the total number of haemocytes (Joseph and Philip, 2007; Pan et al., 2008), however in the current experiment sub-adult prawns held under stress had increased THC and GHC, 96 h post-

transfer. Therefore, the acclimation time of 96 h may be sufficient time for prawns to acclimatise to the stress of acute decreases in water temperature and salinity, by increasing THC and GHC by haematopoiesis, to pre-stress counts (van de Braak, 2002). On the contrary, previous research (Chapter 3) assessing the immune response of *P. monodon* after an acute decrease in water temperature and salinity recorded decreased THC and GHCs, when compared to non-stress controls. Therefore, in the current study increased THC and GHC responses would be plausible, or at least a return to the same mean values as the non-stress group. However, the immune responses were measured after $\dot{M}o_2$ measurements including AMR, which was determined by chasing individual prawns until exhaustion, which may explain the decrease in THC and GHC.

In the current study prawns held under non-stress conditions had significantly lower PO activity. However, a decrease in PO activity is commonly observed during periods of environmental stress as a result of decreased immune vigour (Le Moullac and Haffner, 2000; Perazzolo et al., 2002). The ProPO system is located within the plasma of small and large granular haemocytes; therefore a decrease in GHC causes a decrease in PO activity. These results demonstrate that measuring the immune response of *P. monodon* after prior chasing to exhaustion, may not provide an accurate immune response measure, however as all animals were dealt in the same manner, data are relevant across treatments. Due to a limited number of prawns available in the current study, all measurements were conducted on all prawns. In future experiments potentially compromised prawns, such as prawns used for $\dot{M}o_2$ measures, should not be considered for assessing immune response variables. Further research of non-diseased prawns is necessary for assessing the effects of dietary fucoidan on the A-SCOPE of prawns.

4.8 Conclusion

In general, juvenile prawns fed fucoidan had a higher AMR. Diet had no effect on the $\dot{M}o_2$ rates of sub-adult prawns. However $\dot{M}o_2$ rates were lower in prawns under environmental stress. Prawns held under environmental stress also had increased immune responses, when compared to non-stress groups. Future studies to assess fucoidan in *P. monodon* feeds should explore the potential immunostimulant benefits of fucoidan after a viral disease challenge with white spot syndrome virus and how this virus impacts survival and immune response variables.

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The effect of immunostimulants and inoculation methods on the growth performance, immune response and survival in *Penaeus monodon* challenged with white spot syndrome virus

CHAPTER V

5.1 Abstract

This study investigated the use of immunostimulants FV90 and UP85 (Fucoidan) and Sanictum® (β-glucan and peptidoglycan) in juvenile *Penaeus monodon. P. monodon* were fed feed treatments for 28 days to determine growth performance. Thereafter, two experiments were conducted; the first experiment determined the immune response (THC, GHC and PO activity) of prawns 24 h post-infection with white spot syndrome virus (WSSV), before the second experiment where prawns were inoculated with WSSV to determine effect of diet on survival. In both disease experiments, WSSV was delivered via two infection routes, intramuscular injection (IM) and reverse gavage (RG), while PBS served as controls. These experiments were conducted at a research facility (BIOTEC) in Thailand due to Australia being disease free of WSSV. In the growth experiment the weight gain (WG) specific growth rate (SGR) and survival were not significantly different between treatments. At the conclusion of the growth experiment the THC, GHC and PO activity were not significantly between feed treatments. For the immune response experiment, no interactions between feed treatments and inoculum type on THC were determined 24 h post infection with WSSV via IM. However, THC pooled by treatment determined that *P. monodon* inoculated with WSSV had significantly less THC and GHC when compared to PBS controls. A significant interaction between feed treatment and inoculum type was determined for PO activity when inoculated via IM. Prawns fed the basal and FV90 treatments had significantly lower PO activity than the PBS control group. When *P. monodon* were inoculated via RG there was a significant interaction between feed and inoculum treatments. Prawns fed the UP85 treatment had significantly less THC when infected with WSSV than prawns fed the basal, FV90 and UP85 treatments and inoculated with PBS. However, GHC in RG inoculated treatments were not different, when pooled by inoculum type,

prawns inoculated with WSSV had significantly less GHC, although PO activity was not different. For the challenge experiment, prawns infected with WSSV via IM and fed the basal treatment suffered 100% mortality by day 7, however all immunostimulant treatments had 11.11% survival. In RG groups, survival was high at day 21 for all treatments, although not significantly different between treatments. The viral load for IM infected treatments was not significantly different, while the viral load in the RG treatments was below the level of sensitivity of the molecular assay. Prawns fed the immunostimulant products suffered no negative effects on growth performance or survival during the feeding experiment. The RG method protocol did not elicit a detectable infection level by PCR in the survival challenge experiment and further improvement is required for conducting disease challenges using a more natural infection route and for assessing immunostimulant products which show promise of viral adsorption with the digestive tract.

Introduction

The global aquaculture production of penaeid prawns is adversely affected by outbreaks of viral pathogens within the culture environment. These mortality events cost prawn farmers and farming communities significant financial losses (Lightner and Redman, 1998). White spot syndrome virus (WSSV) is of major importance and is considered one of the most significant viral pathogens, which causes mass mortality in prawns and has affected prawn aquaculture worldwide since 1992 (Lightner, 2003; Flegel et al., 2008). Acute WSSV infected prawns can exhibit gross signs of the disease, such as white spots or reddening of the carapace and appendages and up to 100% mortality occurs within 3 to 10 d (Chou et al., 1995; Lightner, 1996; Lo et al., 1996). However, during experimental infection studies, the white spots on the carapace are not always present, this is thought to be due to mortality occurring much sooner than under commercial conditions (Lightner, 1996; Escobedo-Bonilla et al., 2007). In commercial culture the host is inoculated with the pathogen by means of natural infection; where the route of infection is by cohabitation with infected animals that shed the virus or by ingestion of infected material, such as prawn tissue or faeces (Lotz and Soto, 2002). The target organs of the pathogen include the foregut, midgut, hindgut, gills, antennual gland, integument, muscle, gonads, nervous tissue, lymphoid organ, haematopoietic tissues, heart and haemocytes (Chang et al., 1996; Di Leonardo et al., 2005; Escobedo-Bonilla et al., 2007; Rahman et al., 2008).

Most prawn farming nations with the exception of Australia experience outbreaks of WSSV. While there is no cure for the disease; current methods to reduce outbreaks include chlorination of incoming water and ponds (Lakshmi et al., 2013), broodstock screening using molecular detection techniques (Lightner and Redman, 1998), increasing water temperature (Rahman et al.,

2007) and salinity (Joseph and Philip, 2007), and incorporating high levels of biosecurity at production sites (Lightner, 2003).

Immunostimulants have shown great potential for increasing prawn survival to viral pathogens (Sakai, 1999; Ringo et al., 2012). Immunostimulants, such as fucoidan have demonstrated to increase survival post-infection and immune response in prawns to WSSV (Chotigeat et al., 2004; Immanuel et al., 2012). The mechanism of how fucoidan increases disease resistance in penaeid prawns is unknown. However, it is thought that fucoidan acts similarly to that in human host cells, where inhibition of viral adsorption has been achieved *in-vitro* (De Somer et al., 1968; Baba et al., 1988). Therefore fucoidan may have bioactive characteristics other than increasing immune responses in prawns. Fucoidan has demonstrated to increase the immune response of penaeid prawns, these include increases in; total haemocyte counts (THC), granular haemocyte counts (GHC), phenoloxidase activity (PO activity), respiratory burst activity, superoxide dismutase and phagocytic activity (Traifalgar et al., 2010; Immanuel et al., 2012; Kitikiew et al., 2013). Peptidoglycan (PG) is derived from the cell wall of a Gram positive bacterium, PG has demonstrated immunostimulant properties including increased THC, PO activity, superoxide anion and bactericidal activity in *P. monodon* (Purivirojkul et al., 2006) and phagocytosis and survival in *Marsupenaeus japonicus* challenged with WSSV (Itami et al., 1998). β-glucan is another commonly researched immunostimulant product which has demonstrated increased immune response (THC, PO activity, respiratory burst, superoxide dismutase and lysozyme activity) and resistance to WSSV when fed to *Litopenaeus vannamei* (Bai et al., 2014) and *P. monodon* (Chang et al., 2000).

To determine the susceptibility of host species to pathogens such as WSSV, the host is inoculated with a known concentration of infectious material, this ensures the pathogenicity is the same between animals, and groups of animals can be compared to other treatments (Escobedo-Bonilla et al., 2005). The inoculation methods that are currently used for determining the pathogenicity of a pathogen to a prawn host include; intramuscular injection (IM) (Jiravanichpaisal et al., 2001; Pan et al., 2008; Immanuel et al., 2012), *per os* by feeding infected tissue (Chang et al., 1996; Srisuvan et al., 2006; Joseph and Philip, 2007; Sarlin and Philip, 2011; Bai et al., 2014), immersion (Chotigeat et al., 2004; Huynh et al., 2011), oral intubation (Escobedo-Bonilla et al., 2005; Escobedo-Bonilla et al., 2007) and anal intubation (reverse gavage, RG) (Aranguren et al., 2010). To date there are standardised inoculation methods for IM and oral intubation techniques, and virus titre is quantified by using a syringe or automatic pipette (Escobedo-Bonilla et al., 2005). To determine whether fucoidan inhibits WSSV adsorption in the digestive tract of prawns, the less utilised technique RG, may eliminate problems derived with oral intubation; which include hard structures (mandibles), sharp bends in oral cavity and potential fissures in the epicuticle layer of the foregut due to pipetting pressure, allowing virus to move freely to target cells such as epithelial cells (Escobedo-Bonilla et al., 2007; Aranguren et al., 2010).

Further understanding of the mode of action of fucoidan and the effects on viral adsorption will further benefit our understanding of how to better utilise fucoidan as an immunostimulant to enhance survival in prawn farming. By inoculating *P. monodon* by RG, the viral suspension will be delivered directly to the midgut, reflecting a natural *per os* infection. To compare with

fucoidan, a commercial immunostimulant product Sanictum® (Chemoforma Ltd), containing the active components β-glucan and peptidoglycan will be included as a reference.

The aims of this study were:

- To determine whether fucoidan and Sanictum® enhance growth rates of juvenile *P. monodon*.
- To determine whether the use of reverse gavage (RG) is a better challenge model for viral adsorption than intramuscular injection (IM).
- To measure the immune response (THC, GHC and PO activity) and survival of *P. monodon* fed fucoidan, prior to a WSSV challenge, in comparison to prawns fed a β-glucan (Sanictum®).

Materials and methods

Prawn stock

Experiments from previous research chapters have been conducted in Tasmania, Australia. However, Australia has a disease free status for significant viruses affecting prawn farming including; Taura Syndrome Virus (TSV), White Spot Syndrome Virus (WSSV) and Yellow Head Virus (YHV). Therefore, these experiments were conducted at Shrimp Biotechnology Business Unit (SBBU), located at the Thailand Science Park, Pathumthani, Thailand.

Fifteen day old black tiger prawn (*P. monodon*) postlarvae (PL15) were obtained from a commercial hatchery in Thailand (Siam Black Tiger Shrimp) and transported to Shrimp Biotechnology Business Unit (SBBU), located at the Thailand Science Park, Pathumthani, Thailand. Prawns were on-grown to approximately 2 g in 2000 L tanks within a recirculating seawater system before grading into similar size classes. During this time prawns were fed a commercial prawn feed (Charoen Pokphand Feeds (CPF), 38% crude protein, 5% fat), four times daily (08:00, 11:30, 15:00 & 19:00) to satiation.

Postlarvae were maintained at 15‰ salinity (ATAGO S-10E), 12:12 (light/dark) photoperiod; temperature: 28-32°C (YSI Pro 20) and dissolved oxygen > 5.0 ppm (YSI Pro 20). Water quality parameters were determined by: phenol-hypochlorite method (NH_3: < 0.03 ppm), colorimetric absorbance (NO_2: < 1.0 ppm, NO_3: < 20 ppm), alkalinity by titration (100-150 mg$CaCO_3$/L) and pH was 7.5-8.2 (Eutech Cyberscan pH 11).

To ensure PL were free of disease, pleopods from 20 PL from the stock population were sampled, samples were pooled for viral analysis and screened for viral pathogens common in Thailand (TSV, WSSV, YHV,MBV and IHHNV), using polymerase chain reaction (PCR) techniques.

5.3.2 Experimental system

The experimental recirculating seawater system comprised of thirty 390 L round conical based fibreglass tanks. Tanks were fitted with mesh netting to stop prawns escaping, and tank outlets were screened to keep prawns within tanks. Water quality parameters (NH_3, NO_2, pH, alkalinity, and dissolved O_2) were measured daily and maintained by biofiltration, UV sterilization and solids removal. Aeration was provided by the addition of three air-stones in each tank. The average water temperature during the growth experiment was 29.58 ± 0.41°C. The average salinity was 15‰ and was adjusted as required by the addition of de-chlorinated tap water or seawater.

5.3.3 Feed formulation

Experimental feeds were formulated at 47% crude protein, 7.3% crude lipid and 16.2 MJ kg^{-1} of energy 'as is' (Table 5.1). The basal feed (control) was formulated to contain 6.0 $g.kg^{-1}$ α-cellulose (Sigma-Aldrich), and was partially or completely substituted with fucoidan (FV90 and UP85) to provide a fucoidan concentration of 1000 $mg.kg^{-1}$ (based on previous experiments) or β-glucan (Sanictum®) to provide 50 $mg.kg^{-1}$ (manufacturer's recommendations). Fucoidan test ingredients were: Maritech® *Fucus vesiculosus* extract 92.6% fucoidan (FV90), Maritech®

Undaria pinnatifida extract 87.6% fucoidan [(UP85) Marinova Pty Ltd, Cambridge, Tasmania Australia], and the β-glucan test ingredient was Sanictum® (Chemoforma Ltd).

Fish meal (Skretting Australia, Cambridge, Tasmania, Australia) and South American krill meal (Ridley Aqua-Feeds, Narangba, Queensland, Australia) were included as sources of protein and lipid, while krill meal was a feed attractant. Wheat flour (Four Roses, New South Wales, Australia) and wheat gluten (MP Biomedicals) were binders. Cholesterol (Sigma-Aldrich) was added at 1 $g.kg^{-1}$ and soy lecithin (Lucas Meyer Eurologistic, IIIinois) at 10 $g.kg^{-1}$. A commercial vitamin and mineral premix was included at 3% as recommended by the manufacturer (DSM Nutritional Products Pty Ltd, Wagga Wagga, New South Wales, Australia) (Table 5.1).

Fish meal was sifted through a 1 mm sieve (Greer and Ashburner, 1-C). Dry bulk ingredients were homogenised in a mixer (Brice Australia, Pty Ltd, VFM-20C) for 20 min before small dry ingredients including test ingredients were homogenised in a subsample of the bulk mix, then returned to the bulk mix and mixed for further 20 min. Lecithin and choline chloride (MP Biomedicals) was dissolved in distilled water and added to the dry mixture and further mixed for 20 min until a crumbly dough could be formed. The dough was then passed twice through a 2 mm die of a commercial pasta maker (ItalPast, Mac 10s) and cut to approximately 3.2 mm lengths. Pellets were steamed for 10 min (Tefal, Steam Cuisine) before being air dried (Forma Scientific, 68732-1) until feeds contained less than 10% moisture. To determine moisture content of feeds, 2 g sub-samples were dried for 2 h at 135°C. Feeds were vacuum packed and air-freighted to SBBU Thailand, where they were stored in airtight bags at -20°C until required.

Table 5.1. Ingredient inclusion as is (g.kg^{-1}) and mean chemical composition (n=2) of experimental feeds (g.kg^{-1} DM) +/- S.E. fed to *Penaeus monodon*.

	Feeds				
Ingredients (as is)	**BASAL**	**FV90**	**UP85**	**SANICTUM**	**CPF**
Fishmeal	500.00	500.00	500.00	500.00	
Wheat flour	320.70	320.70	320.70	320.70	
Krill meal	60.00	60.00	60.00	60.00	
Wheat gluten	70.00	70.00	70.00	70.00	
Test ingredient[1]	0.00	1.08	1.13	0.05	
α-cellulose	6.00	4.92	4.87	5.95	
Lecithin	10.00	10.00	10.00	10.00	
Vitamins & mineral premix[2]	30.00	30.00	30.00	30.00	
Cholesterol	1.00	1.00	1.00	1.00	
Stay C	1.00	1.00	1.00	1.00	
Pigment	0.50	0.50	0.50	0.50	
Banox E	0.20	0.20	0.20	0.20	
Choline chloride	0.60	0.60	0.60	0.60	
Chemical composition g.kg^{-1}					
Dry matter	913.40 ± 1.19	921.83 ± 3.41	910.28 ± 0.01	920.63 ± 1.31	912.98 ± 1.18
Crude protein	486.96 ± 1.04	481.24 ± 3.27	484.26 ± 5.47	477.09 ± 3.71	457.49 ± 3.91
Crude lipid	109.69 ± 1.32	108.74 ± 0.22	107.85 ± 1.48	111.30 ± 1.54	88.98 ± 1.63
Gross energy (MJ.kg^{-1})	19.74 ± 0.01	19.43 ± 0.01	19.63 ± 0.01	19.49 ± 0.03	18.40 ± 0.12
Ash	108.12 ± 0.21	112.02 ± 0.37	107.54 ± 0.33	109.33 ± 0.11	133.52 ± 0.17
Protein:energy (g CP.MJ GE^{-1})	24.67	24.77	24.67	24.48	24.86

Feeds contain: BASAL = Control, FV90 = Maritech® *Fucus vesiculosus*, UP85 = Maritech® *Undaria pinnatifida*, SANICTUM = Sanictum®, CPF = Charoen Pokphand Foods.

[1]Fucoidan ingredients (Marinova Pty Ltd) and β-glucan and peptidoglycan ingredient (Chemoforma Ltd).

[2] Vitamin and mineral premix, RAP prawn vitamin/mineral premix, YG12202001 (DSM Nutritional Products Pty Ltd).

5.4 Growth experiment

5.4.1 Acclimation period

Thirty *P. monodon* of similar size were stocked into each tank of the experimental system. Prawns were fed to satiation on a basal feed (control) or a reference feed (CPF) four times per day (0800, 1130, 1500 and 1900 h) for 7 d. Prior to the 0800 h feed, all uneaten pellets, faeces and exuvia were siphoned from tanks. During the 7 day acclimation period any dead animals were replaced with animals from the same cohort.

5.4.2 Experimental period

At the start of the experiment all prawns were individually weighed by placing prawns on a balance (OHAUS, Pioneer™). Thirty prawns (mean weight 3.01 ± 0.63 g) were returned to each of the thirty tanks. Experimental feeds were assigned to tanks using a complete randomised design. Five feed treatments were fed; these consisted of four experimental feeds with seven replicates per treatment and one commercial reference treatment with two replicates. The reference feed was included to compare the growth performance and feed efficiency of prawns fed the reference feed in previous experiments conducted at the facility, although the reference feed was not included in the statistics. Prawns were fed four times daily as per the protocol during the acclimation period and feeding concluded at day 28. Thereafter prawns were fasted for 12 h before three intermoult prawns per tank (n=21 / feed treatment) were removed and sampled for haemolymph.

5.4.3 Immune response

Haemolymph (400 µl) was removed from the ventral sinus using a 26 G needle and 1 ml tuberculin syringe containing 300 µl of chilled AC-1 anticoagulant (450 mM NaCl, 10 mM glucose, 10 mM citric acid, 26 mM sodium citrate, 10 mM EDTA, pH 4.6) (Soderhall and Smith, 1983). The haemolymph-AC1 solution was immediately ejected into a 2 ml microcentrifuge tube and mixed well before storing on ice. After each prawn was sampled, they were placed back into their corresponding tanks within floating cages and fasted for a further 12 h. Thereafter all prawns from each tank were individually weighed to determine the final body weights at the conclusion of the growth experiment. Final weight and total number of surviving prawn data were used to calculate growth performance and survival indices.

5.4.3.1 Total haemocyte count (THC)

One hundred microliters of haemolymph mixture was removed by pipette from the haemolymph sample previously collected. One drop of haemolymph mixture was placed on a Neubauer Improved Bright-line haemocytometer (BOECO) under 400x magnification of a light microscope (Motic, BA300). At least 200 haemocytes were counted in 5 medium squares of the haemocytometer in duplicate. Haemocytes were quantified by multiplying the average number of cells by a conversion factor (2.5×10^5) and multiplying by a correction factor taking into account the anticoagulant volume.

5.4.3.2 Granular haemocyte count (GHC)

From the 100 µl of haemolymph mixture 20 µl was added to 20 µl of formalin and left for 20 min, then 50 µl of Rose Bengal solution (Sigma, 1.2% w/v Rose Bengal in 50% ethanol) was

added to the micro-centrifuge tube and incubated at room temperature for 20 min (Sritunyalucksana et al., 2005). Blood smears were made in duplicate; each smear was made by placing one drop (20 µl) of stained haemolymph solution onto a clean microscope slide, and smeared. Smears were air-dried, then counterstained with haematoxylin (Sigma) for 7 - 10 min, before rinsing in tap water for 10 min followed by immersion for 1 min in 95% ethanol (10 dips) and 100% ethanol (10 dips). Following dehydration, slides were placed in xylene for 5 min and cover slip mounted with Permount. Granular haemocyte counts (GHC) were determined by counting the proportions of granular (small-granular and large-granular) haemocytes in 200 total haemocytes per smear under 400 x objective of a light microscope (Motic, BA300) and expressed as: count/200 x THC (Sritunyalucksana et al., 2005).

5.4.3.3 Phenoloxidase (PO) activity

Haemolymph (300 µl) was removed from haemolymph mixture and centrifuged at 4,000 rpm for 2 min at 4°C. After centrifuging, the plasma was removed from the microcentrifuge tube and 300 µl of cacodylate buffer (Sigma-Aldrich, pH 7.4) was added to the haemocyte cell pellet. Thereafter, the haemocyte-cacodylate mixture was sonicated (Bandelin, Sonorex Digitec DT514) at 30 amplitude for 10 min prior to centrifuging at 16,000 x *g* for 10 min at 4°C to separate the haemocyte lysate from the cell debris. The haemocyte lysate was used for PO activity and protein analyses.

Phenoloxidase activity was determined from haemocyte lysate using a modified method from Supamattaya et al. (2000). For each sample 50 µl of 1% (1 $mg.ml^{-1}$ cacodylate buffer) trypsin (Sigma-Aldrich) was added to 3 wells of 96 well micro-plate (Sterilin™, 612F96) containing 50

µl of haemocyte lysate. The samples were mixed by shaking on a microplate reader before incubating the plate for 10 min at 25°C. Triplicate wells containing cacodylate buffer (50 µl) and trypsin (50 µl) served as the blank. After the incubation period, 50 µl of dihydroxyphenylalanine (L-DOPA) was added to each well, as a substrate and incubated for another 10 min at 25°C. Thereafter the absorbance was measured using a microplate reader (Molecular Devices, SpectraMax M5) at 490 nm every 2 min for a total of 20 min. The change in absorbance was determined over the 20 min period and expressed as rate of change per min, minus the absorbance of the blank. The PO activity was expressed as the increase in absorbance (O.D) 0.001 per min (Δ Abs.min^{-1}) per 100 µl of haemolymph, as a comparison to previous thesis chapters.

5.5 Immune response to WSSV

Postlarvae (mean weight 2.82 ± 0.02 g) were fed experimental feeds as per the protocol used in the growth experiment (section 5.3) for 28 d. At the conclusion, forty prawns of approximately 8.0 g in weight from each experimental feed treatment were removed from growth experiment tanks and allocated to four 100 L glass static aquaria (filled to 70 L) and fasted for 24 h. Each of the four tanks was allocated a different treatment group (Table 5.2). The WSSV stock was prepared following the method of Escobedo-Bonilla et al. (2005) and Rahman et al. (2006). The pathogenicity of the stock concentration was previously determined by technical staff at SBBU, in order to achieve a median lethal dose (LD_{50}) following the method of Reed and Muench, (1938).

Prawns in group 1 were inoculated with WSSV (2.81 x 10^8 copy/ml^{-1}) by intramuscular injection (IM) into the 3rd abdominal segment using a 1 ml syringe and 23 G needle containing 100 µl of WSSV suspension (Table 5.2). Prawns in group 2 were inoculated with WSSV by reverse gavage (RG), where 100 µl of WSSV suspension was administered using an automatic pipette via the anus, the pipette tip was removed prior to releasing pipette actuator and the hindgut was held with light pressure for 5 seconds directly after WSSV inoculum was ejected (Table 5.2). Prawns in group 3 were inoculated with phosphate buffered saline (PBS) by IM at the 3rd abdominal segment as previously described in group 1 prawns, and served as the control for the injection method (Table 5.2). Prawns in group 4 were inoculated with PBS by RG as previously described in group 2 prawns, and served as the control for the RG method (Table 5.2). Twenty four hours later, five prawns per tank were sampled for THC, GHC and PO activity to determine immune response as per section 5.3.3.

Table 5.2. Treatment groups for *Penaeus monodon* for inoculation type and inoculation method for immune response and challenge experiments to WSSV.

Group number	Inoculation type	Inoculation method
1	WSSV	IM
2	WSSV	RG
3	PBS	IM
4	PBS	RG

WSSV = White spot syndrome virus
PBS = Phosphate buffered saline
IM = Intramuscular injection
RG = Reverse gavage

5.6 Survival after infection with WSSV

An experiment was conducted to determine whether fucoidan and Sanictum increase survival of *P. monodon* infected with WSSV, by inoculating prawns using IM and RG inoculation

techniques, outlined in section 5.4. The experiment started 52 d after the initial growth experiment; 108 prawns from each feed treatment (excluding CPF) were randomly allocated into 4 groups (group 1, 2, 3 & 4) as per the previous viral challenge experiment. Prawns from each group were then divided into three glass aquaria (100 L) which served as replicate units (n = 3), therefore each replicate tank consisted of 9 prawns. Thereafter, prawns were fasted for 24 h to ensure digestive functions did not influence viral inoculum evacuation, especially in the RG treatment. At the start of the experiment prawns in groups 3 and 4 were inoculated with PBS using the same protocol and inoculum concentrations and volumes as per section 5.4. Thereafter prawns from groups 1 and 2 were inoculated with WSSV. Twenty four hours later prawns were fed the same experimental feeds as per the feeding experiment, four times daily (0800, 1130, 1500 & 1900). Uneaten feed and exuvia were removed before the 0800 feed and a water change was performed daily to keep water quality parameters within ideal ranges. Survival was recorded up to day 7 for IM treatments and day 21 for RG, the longer duration in the RG treatments was due to slower mortality rates. Pleopods from 3 dead or moribund prawns per WSSV IM and RG replicates were sampled and WSSV was quantified using real-time PCR.

Samples for WSSV quantification were conducted as per the manufacturer's recommendations (EzeeGene® WSSV Real-time PCR kit). Briefly, approximately 25 mg of pleopod from each prawn was ground in a micro-centrifuge tube containing 500 µl lysis buffer and incubated at 95°C for 10 min. Samples were centrifuged at 12,000 rpm for 10 min before removing 200 µl of top layer supernatant and gently mixed with 400 µl of absolute ethanol. Samples were centrifuged at 12,000 rpm for 5 min, prior to decanting ethanol and washing pellet with 500 µl of 75% ethanol. The remaining pellet was air-dried at room temperature for 15-20 min. Thereafter,

the pellet was dissolved using DNase/RNase free treated-water (DEPC) and stored at -20°C until required. Sample DNA was standardised prior to PCR amplification. The reaction was conducted using a commercial WSSV PCR kit (EzeeGene®, WSSV Real-time PCR kit) and PCR conditions were 50°C for 5 min, 95°C for 20 sec followed by 40 cycles 95°C for 3 sec and 60°C for 30 sec. Three positive controls were used ranging from 2×10^2, 2×10^3 and 2×10^4 copies, while samples were considered positive at 20 copies and higher.

5.7 Chemical analyses

Sub-samples of experimental feeds were freeze-dried (Dynavac, FD3) to a constant weight, and chemical composition analysed in triplicate at the Nutrition Laboratory (University of Tasmania, Launceston, Tasmania). Feeds were homogenised to a fine powder using a bench-top hammer mill (Culatti micro hammer-cutter mill, MFC). Samples were wrapped in aluminium foil and placed in an airtight plastic bag at -20°C until required.

Crude protein analysis was conducted using Kjeldahl (FOSS Kjeltec™ 8100), and was calculated as N x 6.25, crude lipid (Bligh and Dyer, 1959), gross energy by bomb calorimetry (Gallenkamp Autobomb, CAB101), ash by combustion (SEM 102C muffle furnace) at 600°C for 2 h, using standard laboratory methods in accordance with AOAC (1995). The moisture content of feeds was determined by drying a 2 g sample of feed in triplicate at 135°C to a constant weight.

5.8 Calculations

Feed intake (FI) (g DM d^{-1}) was calculated as total feed consumed (g dry matter).

FI = ((consumed $W_{feed\ DM}$ (g) – M_{loss} (g)) / 100) x d^{-1}

Where: $W_{feed\ DM}$ = total dry weight of feed consumed (g), M_{loss} = pellet moisture loss (g), d^{-1} = per day

Weight gain was calculated as:

Weight gain (g) = (W_{final} (g) – $W_{initial}$ (g))

Where: FW_{wet} = final wet weight, IW_{wet} = initial wet weight.

Specific growth rate (SGR) was calculated as:

SGR (% d^{-1}) = ((ln (W_{final}) – ln ($W_{initial}$)) / d x 100

Where: W_{final} = mean final wet weight (g), $W_{initial}$ = mean initial wet weight (g), d = no. of days (Ricker, 1979).

Average weekly gain (AWG) was calculated as:

AWG = (W_{final} (g) – $W_{initial}$ (g)) / wk

Where: W_{final} = mean final wet weight (g), $W_{initial}$ = mean initial wet weight (g), wk = duration of experiment in weeks.

Feed efficiency ratio (FER) was calculated as:

FER = (W_{gain} (g) / FI (g)) x 100)

Where: WT_W = mean wet weight gain (g), FI = feed intake (g DM.d^{-1}).

Total haemocyte count (THC cells.ml^{-1}) was calculated as:

THC = (MHC x 2.5 x 10^5) x DF

Where: MHC = mean no. of haemocytes per medium square of haemocytometer, 2.5 x 10^5 cells.ml = conversion factor, changing millimetres to millilitres, DF = dilution factor of the addition of fixative and stain (Sritunyalucksana et al., 2005).

Granular haemocyte count (GHC cells.ml^{-1}) was calculated as:

GHC = (GH x THC)

Where: GH = mean number of small and large granular haemocytes, THC = mean number of haemocytes per ml of haemolymph (Sritunyalucksana et al., 2005).

5.9 Statistical analyses

For the growth experiment, tanks were considered as the replicate unit for assessing growth performance, feed intake, survival and immune response data (after growth experiment). For the immune response to WSSV experiment individual prawn data were considered a single replicate unit. For the survival after challenge with WSSV experiment, individual aquaria data were regarded as a single replicate unit. Test of equal variances were assessed using Levene's test of equality and residual plots. Where data violated equal variances data were $\log_{10}$ transformed.

All statistical analysis and data transformations were conducted using IBM SPSS STATISTICS (version 21). Means were compared by one-way analysis of variance (ANOVA) for comparisons between growth experiment variables. Interactions between feed treatment and inoculum type for immune response variables were compared by two-way ANOVA, when no interactions were present data were pooled by inoculum type and analysed by one-way ANOVA. Results were

considered significant if $P < 0.05$ using a Tukey's HSD test. Survival data post-challenge with WSSV, were compared using a Kaplan-Meier analysis and comparisons were made using a Log Rank (Mantel-Cox) test. Tables and figures were constructed using Microsoft Office Excel 2010, Sigma Plot, version 11.0 (Systat Software, Inc) and GraphPad Prism, version 5.0.

5.10 Results

5.10.1 Growth performance

The average starting weight of prawns in the growth experiment were 3.01 ± 0.63 g (F = 0.055, df = 3, 24, $P = 0.983$) (Table 5.3). At the conclusion of the experiment there was no difference in final weight between prawns fed experimental treatments (F = 0.201, df = 3, 24, $P = 0.894$) and, pooled mean weights were 7.42 ± 0.19 g (Table 5.3). In contrast prawns fed the reference treatment had an average final weight of 6.45 ± 0.42 g. The FI of prawns fed the FV90 treatment (7.93 ± 0.36 g DM d^{-1}) was significantly higher (F = 3.808, df = 3, 24, $P = 0.023$) than prawns fed the basal treatment (6.71 ± 0.26 g DM d^{-1}), on average prawns fed FV90 consumed 1.22 g DM d^{-1} more compared to prawns fed the basal treatment, although not different to other treatments (Table 5.3). The survival rates of prawns fed all experimental treatments were not significantly different (F = 0.504, df = 3, 24, $P = 0.683$) between treatments; the pooled average survival rate was 86.2 ± 2.26% (Table 5.3). There was no difference in FER of prawns fed experimental treatments (F= 1.128, df = 3, 24, $P = 0.357$), FER ranged from 45.67 ± 2.02% (FV90) to 49.96 ± 1.78% (basal). The SGR and AWG of prawns fed experimental treatments were not significantly different (F = 0.196, df = 3, 24, $P = 0.898$ and F = 0.188, df = 3, 24, $P = 0.904$), on average the SGR was 3.21 ± 0.08% d^{-1} and the AWG was 1.11 ± 0.04 g wk^{-1}.

Table 5.3. Mean growth performance indices and survival (± S.E.) of sub-adult *Penaeus monodon* fed feeds containing different sources of fucoidan and β-glucan after 28 d (df = 3, 24).

	Units	Feeds					One-way ANOVA	
		BASAL	**FV90**	**UP85**	**SANICTUM**	**CPF**	**F-value**	***P***
Initial weight	g	3.02 ± 0.75	3.02 ± 0.77	3.02 ± 0.79	3.01 ± 0.76	2.97 ± 0.09	0.055	0.983
Final weight	g	7.36 ± 0.18	7.36 ± 0.19	7.53 ± 0.20	7.42 ± 0.17	6.45 ± 0.42	0.201	0.894
Feed intake[1]	g DM d^{-1}	6.71 ± 0.26[a]	7.93 ± 0.36[b]	7.77 ± 0.26[ab]	7.51 ± 0.20[ab]	6.05 ± 0.34	3.808	0.023
Weight gain[2]	g	4.35 ± 0.10	4.35 ± 0.22	4.51 ± 0.14	4.43 ± 0.18	3.48 ± 0.56	0.203	0.894
Specific growth rate[3]	% d^{-1}	3.19 ± 0.04	3.18 ± 0.10	3.26 ± 0.07	3.22 ± 0.09	2.76 ± 0.33	0.196	0.898
Average weekly gain[4]	g wk^{-1}	1.09 ± 0.03	1.09 ± 0.06	1.13 ± 0.03	1.11 ± 0.05	0.87 ± 0.14	0.188	0.904
Feed efficiency ratio[5]	%	49.96 ± 1.78	45.66 ± 2.02	46.73 ± 1.02	48.46 ± 2.11	38.43 ± 5.89	1.128	0.357
Survival	%	83.33 ± 1.92	87.14 ± 2.86	88.24 ± 2.04	86.19 ± 2.23	80.00 ± 0.00	0.504	0.683

Feeds contain: BASAL = Control, FV90 = Maritech® *F. vesiculosus*, UP85 = Maritech® *U. pinnatifida*, SANICTUM = Sanictum®, CPF = Charoen Pokphand Foods commercial feed.

[1]Feed intake (g DM d^{-1}) = (dry matter of feed fed / d^{-1}).

[2]Weight gain (g) = final wet weight – initial wet weight.

[3]Specific growth rate (% d^{-1}) = ln(final wt.) – ln(initial wt.) x 100 / days of experiment.

[4]Average weekly gain (g wk^{-1}) = (wet weight final – wet weight initial / weeks of experiment.

[5]Feed efficiency ratio (%) = (mean wet weight gain (g) / feed intake (g DM) x 100.

Means with different superscripts are significantly different ($P < 0.05$ was considered significant).

CPF (n = 2) are not included in statistics.

5.10.2 Immune response

Growth experiment

After feeding prawns experimental treatments for 28 days there were no significant differences ($F = 1.603$, df = 3, 24, $P = 0.215$) in THC between prawns fed all feeds (Table 5.4). The average number of total haemocytes ranged from 1.80 ± 0.15 cells $ml^{-1} \times 10^7$ for prawns fed FV90 to 2.25 ± 0.19 cells $ml^{-1} \times 10^7$ cells for prawns fed Sanictum. Similarly, when granular haemocytes were quantified there were no significant difference ($F = 2.298$, df = 3, 24, $P = 0.103$) in prawns fed experimental treatments (Table 5.5). The average number of granular haemocytes ranged from 2.05 ± 0.28 cells $ml^{-1} \times 10^6$ for prawns fed FV90 to 2.85 ± 0.55 cells $ml^{-1} \times 10^6$ for prawns fed Sanictum. At the conclusion of the feeding experiment PO activity (Δ in $Abs.min^{-1}$) was not significantly different between treatments ($F = 0.640$, df = 3, 24, $P = 0.597$). PO activity ranged from 275.80 ± 18.83 for prawns fed FV90 to 313.95 ± 24.45 Δ in $Abs.min^{-1}$ for prawns fed UP85 (Figure 5.1).

Immune response to WSSV

After the feeding experiment a total of 20 prawns from each experimental feed treatment were inoculated (n = 5) by intramuscular injection (IM) with either White Spot Syndrome Virus (WSSV) or phosphate buffered solution (PBS), or by reverse gavage (RG) with either WSSV or PBS. PBS served as the control groups.

Intramuscular injection

For prawns inoculated by IM there was no interaction between feed treatments and inoculation type on the THC of prawns at 24 h post-infection, assessed by two-way ANOVA ($F = 1.814$, df

= 3, 32, *P* = 0.164). Therefore, THC data for feed treatments were pooled according to the inoculation type (WSSV and PBS), for prawns inoculated by IM technique. The THC of pooled prawns by inoculum type (WSSV and PBS) was significantly different (F = 31.831, df = 1, 38, *P* < 0.001) at 24 h post-infection, prawns inoculated with WSSV had 56.1% fewer haemocytes (0.89 ± 0.07 cells $ml^{-1} \times 10^7$ when compared to PBS treated prawns (2.04 ± 0.20 cells $ml^{-1} \times 10^7$ (Figure 5.2).

Similarly, there was no interaction between feed treatments and inoculation type on the GHC of prawns at 24 h post-infection (IM), assessed by two-way ANOVA (F = 0.898, df = 3, 32, *P* = 0.453). Therefore, GHC data were pooled according to the inoculation type (WSSV and PBS). The pooled granular haemocytes of prawns was significantly different between inoculum types (F = 92.965, df = 1, 38, *P* < 0.001), prawns inoculated with WSSV had 83% fewer granular haemocytes than prawns inoculated with PBS (Figure 5.3).

After challenging prawns with either PBS or WSSV by IM there was an interaction between feed treatment and inoculation type for PO activity (F = 4.928, df = 3, 32, *P* = 0.006) (Figure 5.4). Prawns fed the basal and FV90 treatments and inoculated with WSSV had a significantly lower PO activity (124.12 ± 12.25 and 158.71 ± 25.65 Δ Abs/min^{-1} than the PBS control groups (403.43 ± 13.66 and 386.07 ± 25.05 Δ Abs/min^{-1} (Figure 5.4). However, prawns fed UP85 and Sanictum were not significantly different between inoculum types (Figure 5.4).

Reverse gavage

There was a significant interaction (F = 2.954, df = 3, 32, *P* = 0.047) of feed treatment and inoculation type on the mean THC of prawns within the RG treatment (Figure 5.5). Prawns fed UP85 and inoculated with WSSV had significantly less haemocytes (0.77 ± 0.17 cells ml^{-1} x 10^7) when compared to prawns fed basal, FV90 and UP85 and inoculated with PBS (2.77 ± 0.41, 2.37 ± 0.32 and 2.68 ± 0.22 cells ml^{-1} x 10^7 respectively) (Figure 5.5). There was no interaction (F = 0.975, df = 3, 32, *P* = 0.417) between feed treatment and inoculation type on the mean GHC of prawns within the RG treatment. The pooled granular haemocytes of prawns was significantly different between inoculum types (F = 14.263, df = 1, 38, *P* = 0.001), prawns inoculated with WSSV had 51% fewer granular haemocytes than prawns inoculated with PBS (Figure 5.6). After challenging prawns with either PBS or WSSV by RG there was no interaction between feed treatment and inoculation type for PO activity (F = 2.347, df = 3, 32, *P* = 0.091) (Figure 5. 7).

Table 5.4. Mean total haemocyte count (THC) (cells ml^{-1} x 10^7 ± S.E.) of *Penaeus monodon* fed feeds containing different sources of immunostimulants after 28 d and 24 h post-inoculation with WSSV.

	After feeding experiment	WSSV IM	PBS IM	WSSV RG	PBS RG
Treatments	**n=7**	**n=5**	**n=5**	**n=5**	**n=5**
BASAL	1.96 ± 0.13	0.82 ± 0.08	2.21 ± 0.63	1.79 ± 0.46^{ab}	2.77 ± 0.41
FV90	1.80 ± 0.15	1.10 ± 0.09	2.71 ± 0.27	2.19 ± 0.36^{b}	2.37 ± 0.32
UP85	2.18 ± 0.11	0.86 ± 0.20	1.47 ± 0.26	0.77 ± 0.17^{a}	2.69 ± 0.23
SANICTUM	2.25 ± 0.19	0.81 ± 0.17	1.79 ± 0.16	1.42 ± 0.20^{ab}	1.83 ± 0.28

Feeds contain: BASAL = Control, FV90 = Maritech® *F. vesiculosus*, UP85 = Maritech® *U. pinnatifida*, SANICTUM = Sanictum®.
After feeding experiment: F-value = 1.603, df = 3, 24, $P = 0.215$.
WSSV injection: F-value = 0.908, df = 3, 16, $P = 0.459$.
PBS injection: F-value = 2.070, df = 3, 16, $P = 0.145$.
WSSV reverse gavage: F-value = 3.514, df = 3, 16, $P = 0.04$.
PBS reverse gavage: F-value = 1.828, df = 3, 16, $P = 0.183$.
Mean values within columns with different superscripts are significantly different ($P < 0.05$ was considered significant).

Table 5.5. Mean granular haemocyte count (GHC) (cells ml^{-1} x 10^6 ± S.E.) of *Penaeus monodon* fed feeds containing different sources of immunostimulants after 28 d and 24 h post-inoculation with WSSV.

	After feeding experiment	WSSV IM	PBS IM	WSSV RG	PBS RG
Treatments	**n=7**	**n=5**	**n=5**	**n=5**	**n=5**
BASAL	2.36 ± 0.41	0.47 ± 0.11	2.77 ± 0.83	2.55 ± 0.79	3.98 ± 0.79
FV90	2.05 ± 0.28	0.46 ± 0.08	3.92 ± 0.88	2.35 ± 0.50	4.46 ± 1.08
UP85	2.38 ± 0.32	0.54 ± 0.19	2.40 ± 0.56	0.70 ± 0.20	3.86 ± 0.41
SANICTUM	2.85 ± 0.55	0.56 ± 0.10	2.91 ± 0.52	1.74 ± 0.58	2.65 ± 0.80

Feeds contain: BASAL = Control, FV90 = Maritech® *F. vesiculosus*, UP85 = Maritech® *U. pinnatifida*, SANICTUM = Sanictum®.
After feeding experiment: F-value = 2.298, df = 3, 24, $P = 0.103$.
WSSV injection: F-value = 0.128, df = 3, 16, $P = 0.942$.
PBS injection: F-value = 0.825, df = 3, 16, $P = 0.499$.
WSSV reverse gavage: F-value = 2.839, df = 3, 16, $P = 0.071$.
PBS reverse gavage: F-value = 0.908, df = 3, 16, $P = 0.459$.
$P < 0.05$ was considered significant.

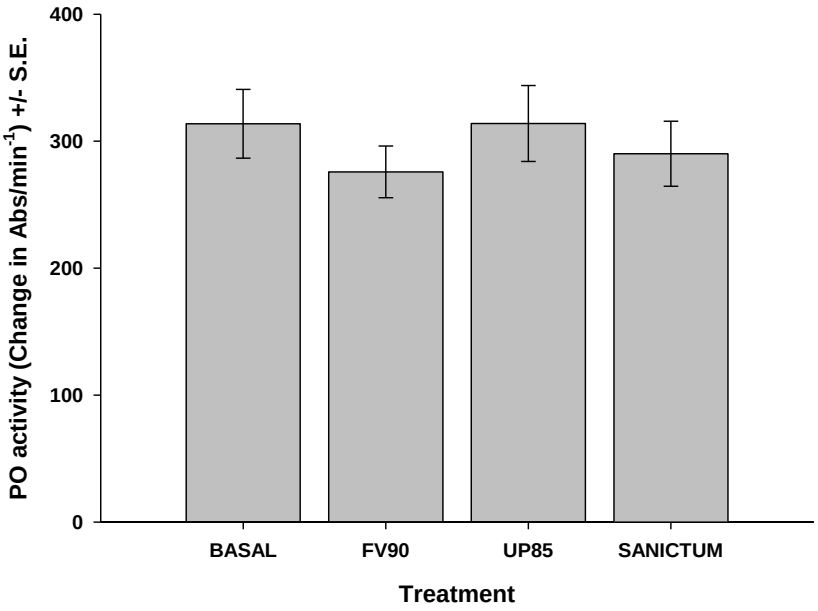

Figure 5.1. Mean phenoloxidase activity (PO activity) (Δ in Abs.min^{-1} ± S.E.) of *Penaeus monodon* fed different diets after 28 d (F = 0.640, df = 3, 24, *P* = 0.597). *P* < 0.05 was considered significant.

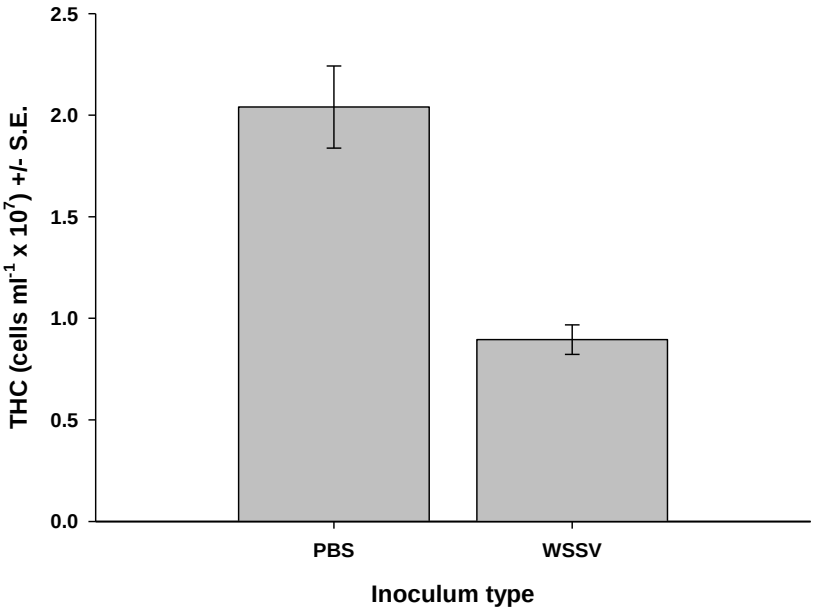

Figure 5.2. Mean total haemocyte counts (THC) (cells ml^{-1} x 10^7 ± S.E.) of *Penaeus monodon* pooled by inoculum type by intramuscular injection (F = 31.831, df = 1, 38, $P < 0.001$). $P < 0.05$ was considered significant.

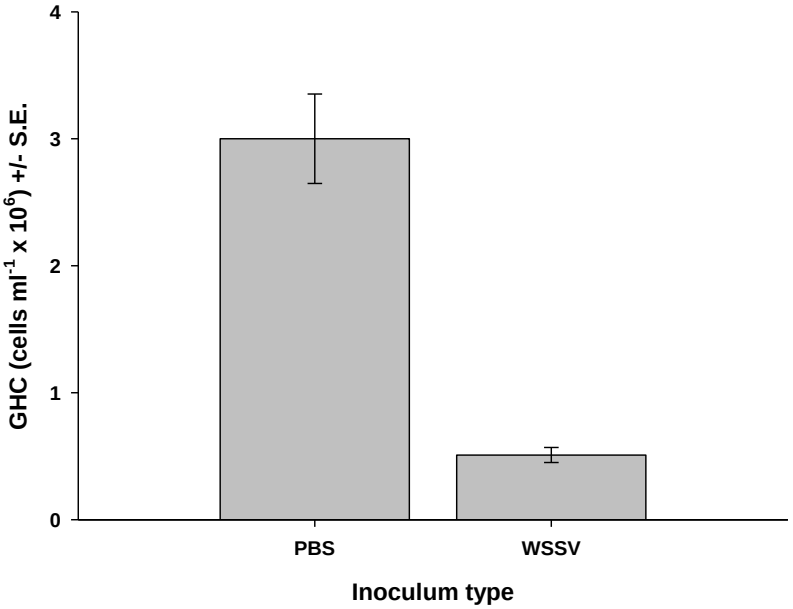

Figure 5.3. Mean granular haemocyte counts (GHC) (cells ml^{-1} x 10^6 ± S.E.) of *Penaeus monodon* pooled by inoculum type by intramuscular injection (F = 92.965, df = 1, 38, $P < 0.001$). $P < 0.05$ was considered significant.

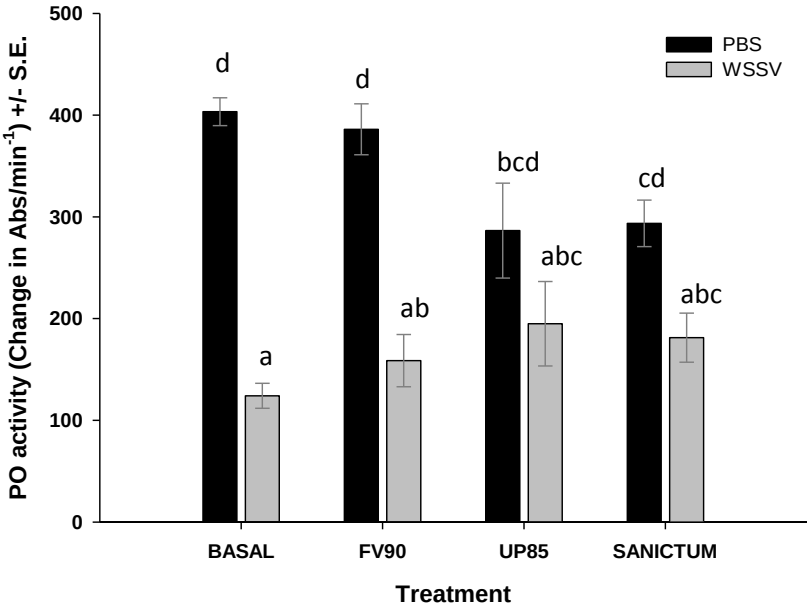

Figure 5.4. Mean phenoloxidase activity (PO activity) (Δ in abs.min^{-1} ± S.E.) of *Penaeus monodon* fed different diets after 28 d and 24 h after inoculation with PBS and WSSV by IM (F = 4.928, df = 3, 32, *P* = 0.006). Mean values with different letters are significantly different (*P* < 0.05 was considered significant).

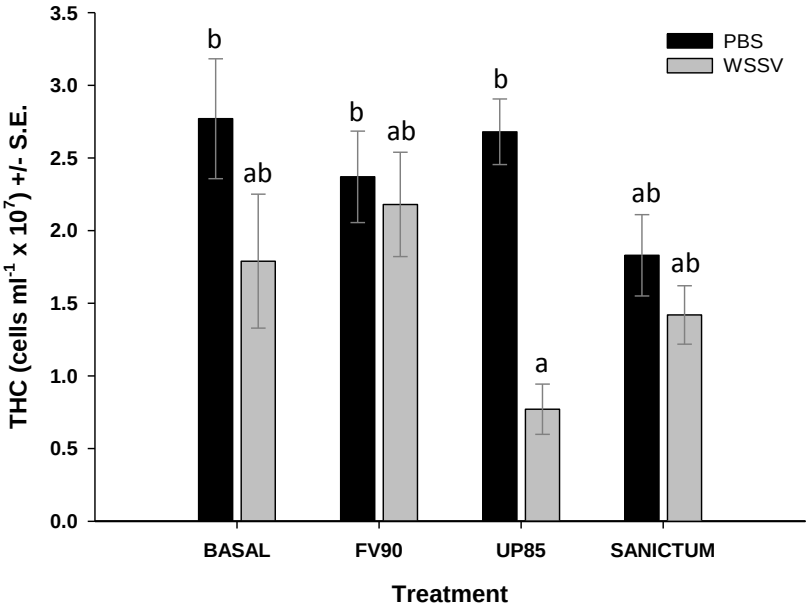

Figure 5.5. Mean total haemocyte counts (THC) (cells ml^{-1} x 10^7 ± S.E.) of *Penaeus monodon* for the interaction between diet and inoculum type using reverse gavage (F = 2.954, df = 3, 32, *P* = 0.047). Mean values with different superscripts are significantly different (*P* < 0.05 was considered significant).

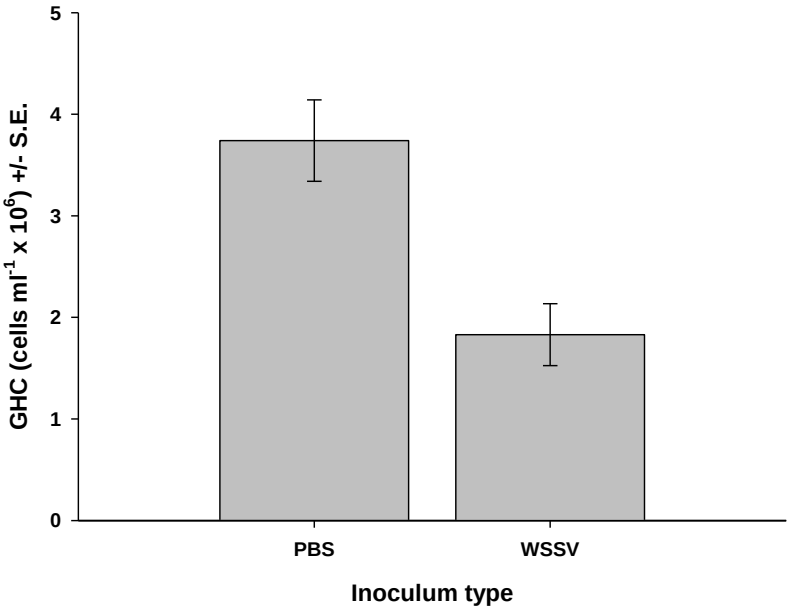

Figure 5.6. Mean granular haemocyte counts (GHC) (cells ml^{-1} x 10^6 ± S.E.) of *Penaeus monodon* pooled by inoculum type by reverse gavage (F = 14.263, df = 1, 38, *P* = 0.001). *P* < 0.05 was considered significant

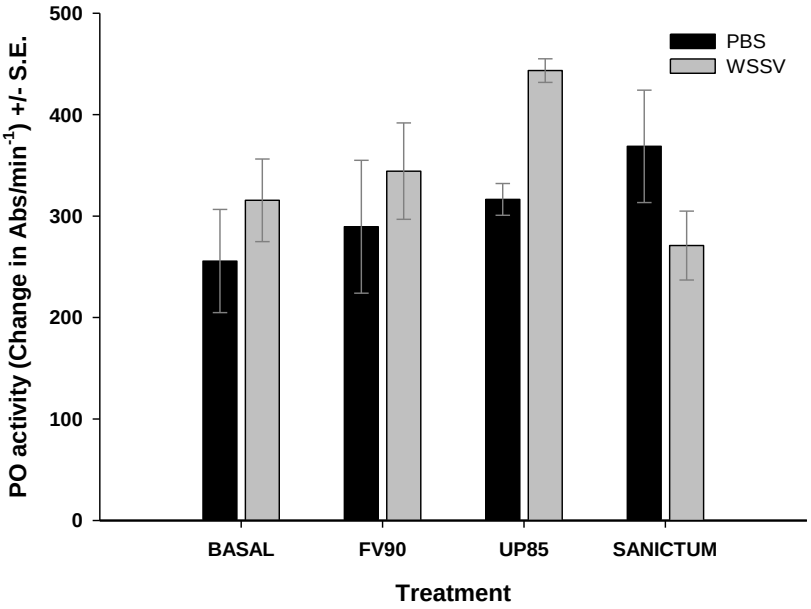

Figure 5.7. Mean phenoloxidase activity (PO activity) (Δ Abs/min^{-1} ± S.E.) of *Penaeus monodon* fed different feeds after 28 d and 24 h after inoculation with PBS and WSSV by RG ($F = 2.347$, df = 3, 32, $P = 0.091$). Values < 0.05 are considered significant.

5.10.3 Survival after challenge with WSSV

During the disease challenge prawns were infected with WSSV via IM and RG inoculation methods (Figure 5.8 and 5.9). A Kaplan-Meier survival analysis was conducted between replicates of prawns fed each feed treatment. For prawns inoculated by IM there were no significant differences between replicates, basal ($\chi2 = 4.636$, df = 2, $P = 0.098$), FV90 ($\chi2 = 0.756$, df = 2, $P = 0.685$), UP85 ($\chi2 = 0.202$, df = 2, $P = 0.904$) and Sanictum ($\chi2 = 0.032$, df = 2, $P = 0.984$). When replicate tanks were pooled by treatment there were no significant differences ($\chi2 = 0.276$, df = 3, $P = 0.964$) in survival for prawns fed all feed treatments and inoculated with WSSV by IM (Figure 5.8). Prawns fed the basal treatment had 0% survival at day 7, while prawns fed all other treatments had 11.11% survival (Figure 5.8). An additional group of prawns inoculated PBS by IM had 100% survival at day 7 (data not shown).

When prawns were inoculated by RG there were no significant differences between replicates, basal ($\chi2 = 1.646$, df = 2, $P = 0.439$), FV90 ($\chi2 = 1.013$, df = 2, $P = 0.603$), UP85 ($\chi2 = 1.987$, df = 2, $P = 0.370$) and Sanictum ($\chi2 = 1.198$, df = 2, $P = 0.549$). When replicate tanks were pooled by treatment there were no significant differences ($\chi2 = 0.566$, df = 3, $P = 0.904$) in survival for prawns fed all feed treatments and inoculated with WSSV by RG (Figure 5.9). Prawns fed the basal treatment had 70.4% survival at day 21, while prawns fed FV90 had 66.6%, and prawns fed UP85 and Sanictum had 63% survival respectively (Figure 5.9). An additional group of prawns inoculated PBS by RG had 100% survival at day 21 (data not shown).

The viral load (WSSV copies ml) of moribund prawns was not significantly different between feed treatments for prawns inoculated using IM ($F = 0.771$, df = 3, 8, $P = 0.542$) (Figure 5.10).

Prawns inoculated by RG recorded either undetectable or viral copies below the sensitivity (20 copies) of the PCR assay and therefore were presumed negative for WSSV.

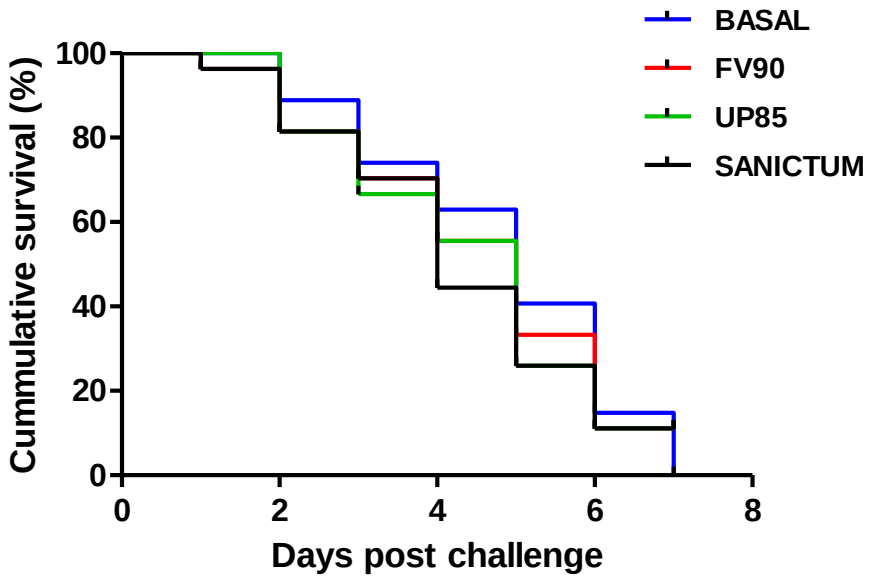

Figure 5.8. Survival of *Penaeus monodon* pooled by diet, after intramuscular injection of WSSV ($\chi 2 = 0.276$, df = 3, $P = 0.964$). $P < 0.05$ was considered significant.

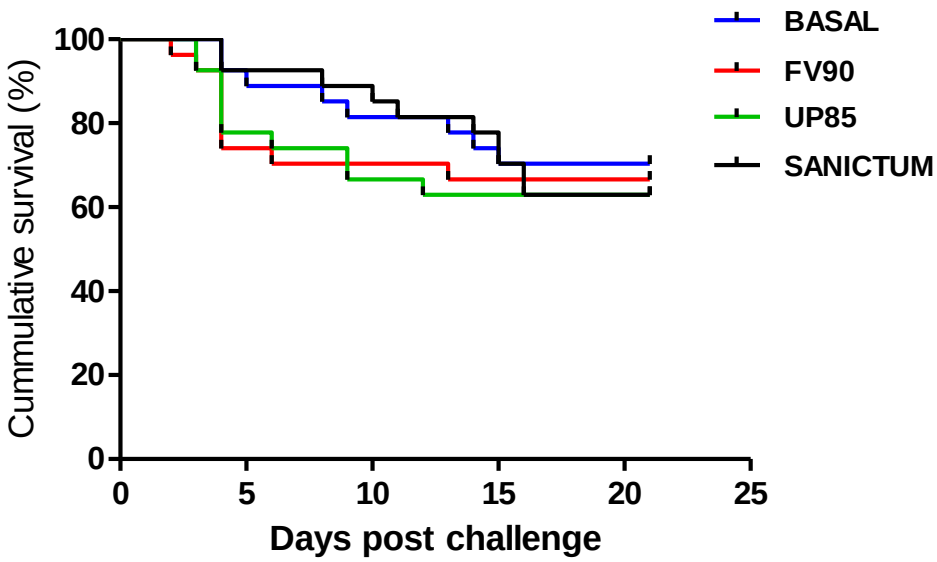

Figure 5.9. Survival of *Penaeus monodon* pooled by diet, after reverse gavage inoculation of WSSV ($\chi 2 = 0.566$, df = 3, $P = 0.904$). $P < 0.05$ was considered significant.

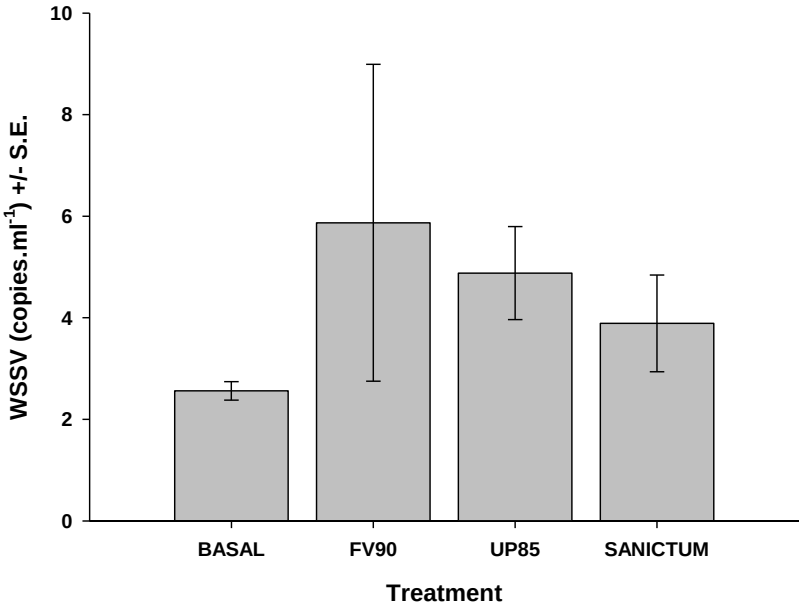

Figure 5.10. Viral load (WSSV copies.ml^{-1} x 10^6 ±S.E.) of *Penaeus monodon* by diet after challenge by IM (F= 0.771, df = 3, 8, $P = 0.542$). $P < 0.05$ was considered significant.

5.11 Discussion

Immune response to WSSV

THC, GHC and PO activity analyses were used to detect differences in immune response prior to and after infection with WSSV for *P. monodon* inoculated by IM and RG inoculation treatments. After feeding different diets for 28 days there were no differences in THC or GHC. When *P. monodon* were infected with WSSV by IM there was a significant decrease in THC (51%) at 24 h post-challenge, when pooled by inoculum type (PBS or WSSV). However, there was no effect of diets. Similarly, when GHC were pooled by inoculum type; the GHC of *P. monodon* followed a significant decrease, where prawns infected with WSSV had 33% less granular haemocytes when compared to the control group. Reductions in THC up to 60% of the pre-infection counts have been reported 24 h after WSSV infection (van de Braak et al., 2000; Chang et al., 2003). *P. monodon* fed diets containing fucoidan (1, 2 and 3 $g.kg^{-1}$) for 45 days demonstrated that THC increased with increasing fucoidan inclusion, when compared to the control group (Immanuel et al., 2012). However, in the same study THC decreased at 10 days post-infection when infected with WSSV, before returning to pre-infection numbers 21 days post-challenge. Furthermore, *P. monodon* suffered 100% mortality by day 21, for prawns in the control group. When *P. monodon* were fed β-glucan for 20 days prior to a WSSV disease challenge there was a significant reduction in THC 24 h post-infection, decreasing by day 6; while prawns fed 2, 10 and 20 $g.kg^{-1}$ β-glucan were the only surviving treatments by day 24 (Chang et al., 2003). In the current experiment THC and GHC decreased in agreement with previous research, which assessed fucoidan (Immanuel et al., 2012) and β-glucan (Chang et al., 2003; Babu et al., 2013). However, in the current experiment haemolymph was only sampled for immune response at one time point, therefore, further comparisons on the effect of time post-infection after 24 h cannot be

determined. Haemolymph was not sampled at other points in the current research due to the increased numbers of prawns required for the analyses and increased expenses for obtaining additional experimental holding systems. However, future research would benefit greatly by incorporating additional sampling times into the experimental regime.

Haemocyte counts are commonly conducted during pathogen infection experiments as haemocytes are an important component for host defence (Soderhall and Cerenius, 1992). The reduction in circulating haemocytes may be due to haemocyte reactions; such as phagocytosis or aggregation at sites with infected host cells or the haemocytes were infected by WSSV (van de Braak et al., 2000). Granular haemocytes were present in greater numbers at infection sites in *P. monodon* infected with WSSV and contain the prophenoloxidase system and active component PO activity (Soderhall and Cerenius, 1996).

When *P. monodon* were infected with WSSV by RG, prawns fed UP85 had significantly less THC 24 h post-challenge, than prawns inoculated with PBS. However, the GHC were not different between diet and inoculum type, therefore low THC for prawns fed UP85 did not manifest a similar decreasing trend in granular haemocytes. When GHC were pooled by inoculum type, prawns infected with WSSV had significantly lower granular haemocytes. This decrease in granular haemocyte numbers suggests that the RG method may have elicited a low level WSSV infection, compared to a higher level of infection when inoculated by IM, and haemocyte aggregation at infection sites may explain this decrease (van de Braak, 2000). Prawns inoculated in the immune response experiment were not analysed for WSSV concentration using PCR, unlike the survival challenge experiment.

In the current study the PO activity (IM) of *P. monodon* 24 h after inoculation with WSSV was significantly lower in basal and FV90 treatments when compared to the PBS control groups, similar to prawns fed UP85 and Sanictum and infected with WSSV. On the other hand, prawns fed UP85 and Sanictum had similar PO activity for both WSSV and PBS treatments, showing a limited or delayed response to the WSSV infection. Higher PO activity values at 24 h post-infection were observed in *L. vannamei* fed *Gracilaria tenuistipitata* and then challenged with the bacterium *Vibrio alginolyticus* and demonstrated an increased immune response and disease resistance 6 days later (Hou and Chen, 2005). However, when *P. monodon* were fed β-glucan treatments prior to a WSSV disease challenge, the PO activity at 24 h post-infection was similar to non β-glucan control prawns, although by day 9 all control prawns had died and β-glucan fed prawns survived to day 24 where PO activity values returned to pre-infection baseline values (Chang et al., 2003). In the current research, this trend in PO activity was not observed in GHC in prawns inoculated with WSSV and may be attributed to individual variation amongst prawns sampled from each feed treatment, and UP85 and Sanictum may provide some additional protection against WSSV at 24 h after inoculation.

Survival after WSSV challenge

The RG method was adopted in the current study to determine whether fucoidan may enhance survival of *P. monodon* by blocking viral adsorption of WSSV within the gut as proposed by De Somer et al. (1968) and Baba et al. (1988). In the WSSV challenge, prawns were inoculated with WSSV in the same manner as the immune response experiment, by IM and RG, using a viral dose of 2.81 $x10^{8}$ copies/ml^{-1}. *P. monodon* infected with WSSV by IM recorded 100% mortality by day 7 for prawns fed the basal treatment, while prawns fed all immunostimulant treatments

recorded 11.11% survival at day 7. On the contrary, when *P. monodon* were inoculated with WSSV by RG, prawns fed the basal treatment had 70.37% survival at day 21, while prawns fed FV90, UP85 and Sanictum had survival rates of 66.67, 62.96 and 62.96% respectively; mortality in all prawns ceased by day 21. Negative controls for IM and RG treatments were inoculated with PBS using the same protocol; survival of IM inoculated prawns was 100% after 7 days and for RG inoculated prawns, survival was 100% after 21 days.

When *P. monodon* were fed fucoidan at concentrations of 1, 2 and 3 $g.kg^{-1}$ for 45 days the survival percentage increased according to fucoidan inclusion (Immanuel et al., 2012). Prawns fed the control diet had 100% mortality by day 10, while prawns fed fucoidan suffered 50% mortality when fed the higher concentration, increasing to 68% when fed 1 $g.kg^{-1}$ (Immanuel et al., 2012). The increase in survival was proposed to be due to the inhibition of fucoidan against WSSV, where the negative charges of the sulfate group in fucoidan bind with the positive charges at the V3 loop of the viral envelope, determined by Haroun-Bouhedja et al. (2000) in human medicine. In the current experiment the mortality rates of IM inoculated prawns was higher than the study by Immanuel et al. (2012), this may be explained by the decreased duration of feeding fucoidan or the pathogenicity of the WSSV inoculum.

When *P. monodon* were fed β-glucan at a graded series of 1, 2, 10 and 20 $g.kg^{-1}$ prior to a WSSV challenge, the mortality rate reached 100% by day 12 when fed 1 $g.kg^{-1}$, while the control group reached 100% mortality by day 9 (Chang et al., 2003). The highest survival rate of 42% was observed at the inclusion rate of 10 $g.kg^{-1}$ by day 24, followed by 30% survival when fed β-glucan at 2 $g.kg^{-1}$ (Chang et al., 2003). The protective role of β-glucan in that study determined the increase in survival was due to β-glucan enhancing immune responses and preventing

components of the immune system from succumbing to the WSSV infection, prior to a gradual recovery (Chang et al., 2003). In the current research the mortality rate was much quicker than that determined by Chang et al. (2003); this may be explained by inclusion level of Sanictum® which was drastically lower, determined by manufacturer's recommendations or the pathogenicity of the WSSV inoculum.

All individual prawn pleopods in the WSSV infected IM and RG groups (challenge experiment) were analysed for viral load using PCR. Prawns inoculated by IM, viral load ranged from 2.56 $x10^6$ for prawns fed basal up to 5.15 x 10^6 copies/ml for prawns fed Sanictum, although there were no significant differences. However, the viral load of prawns inoculated by RG was either undetectable by RT-PCR or below the sensitivity of 20 copies/ml. The control group inoculated with PBS had 100% survival using RG, therefore, mortality is presumed to be due to low infection level or no infection, where virus may have been partially inactivated due to digestive enzymes and chemical barriers, before overwhelming susceptible epithelial cells (Escobedo-Bonilla et al., 2005). It has been proposed that these chemical barriers within the digestive tract may account for a reduction in the severity of WSSV infection by 1 $\log_{10}$ when compared with the IM inoculation method (Escobedo-Bonilla et al., 2005). In the current study tissues and organs were not assessed by histopathology or immuno-histopathology, to determine route of infection in RG treatment, these methods show potential for use in future research (Escobedo-Bonilla et al., 2007; Kulkarni et al., 2013).

Growth performance

The growth performance results indicate that prawns fed the fucoidan and β-glucan treatments were not different at the conclusion of the 28 d feeding experiment. Final weights, SGR, AWG, FER and survival were all similar in prawns fed immunostimulants and the control feed treatments. FI was higher in prawns fed FV90 than prawns fed the basal treatment, however the increased FI did not manifest in higher growth rates or FER. The difference in FI may be explained by the collection of uneaten food; in this experiment uneaten feed was not collected for calculating FI, due to the experimental system design and tank design. In the current research, two additional tanks of prawns were fed the CPF treatment and acted as an internal standard to reference performance at a facility level. However, prawns fed CPF were not included in the statistics due to only having two replicate units. The CPF prawn feed is considered the "gold standard" prawn feed in Thailand. In general, prawns fed the experimental treatments performed better in all measured growth indices, when compared to the CPF treatment. Prawns fed experimental feeds had a mean weight gain of 4.41 g compared to 3.48g for prawns fed the CPF treatment, and prawns fed experimental feeds had a mean SGR and FER of 3.21%.d^{-1} and 47.7% respectively, compared to 2.76%.d^{-1} and 38.43% for prawns fed the CPF treatment.

An increase in growth performance of *Marsupenaeus japonicus* has been recorded when fed a diet containing 0.5 and 1 g.kg^{-1} fucoidan for duration of 56 days (Traifalgar et al., 2010). In that study prawns had an average weight gain of 202.8 and 215.1% respectively and SGR was 1.9 and 2.06%.d^{-1} respectively. In the current study, fucoidan (FV90 and UP85) and β-glucan (Sanictum) fed *P. monodon* had similar weight gain and SGR to the basal fed prawns, on average weight gain was 147% when expressed as a percentage after 28 days, and the average SGR was

3.22%.d^{-1}. Average FCR in the current study was 2.38 for prawns fed fucoidan and β-glucan, while this is higher than the values of 1.8 observed by Traifalgar et al. (2010). In the current study FI does not take into account uneaten feed and is therefore overestimated.

When *L. vannamei* were fed diets containing β-glucan at 1 and 2 g.kg^{-1} for 30 days the highest weight gain was achieved by feeding 1 g.kg^{-1} β-glucan, resulting in an average gain of 5.38 g (Shivananda Murthy et al., 2009). In the current study average weight gain ranged from 4.35 g for prawns fed fucoidan (FV90) to 4.43 g for prawns fed β-glucan (Sanictum). Although these gains maybe lower, the culturing conditions, species and experimental design may account to these differences.

Although no differences were determined in the current study, biological performance was high; fucoidan and β-glucan treatments had no negative effects on growth performance or feed efficiencies.

5.12 Conclusion

In this study growth performance was not enhanced when *P. monodon* were fed fucoidan and β-glucan treatments, when compared to the control, however in all treatments growth and feed efficiencies were high and no negative effects on feed intake or survival were seen. Fucoidan and β-glucan treatments did not provide enhanced protection in *P. monodon* to an infection with WSSV by IM. Prawns inoculated with WSSV by IM showed decreasing THC, GHC and PO activity at 24 h post-infection. It is possible that the dose of fucoidan and β-glucan or duration of feeding may explain results. Prawns inoculated with WSSV by the RG method demonstrated a decrease in THC, when fed UP85 and challenged with WSSV, however did decrease in activity

did not manifest a decrease in GHC or PO activity, when compared to other feed treatments. Prawns inoculated by RG showed a general decrease in GHC when compared to the PBS control, demonstrating an immune response was evident by RG, however no change in PO activity was determined. Prawns inoculated with WSSV by RG demonstrated survival rates of over 60%, indicating the RG method elicited low level disease, however viral loads of dead and surviving prawns were below the sensitivity of the PCR or undetectable.

To date there are standardised disease challenge models for IM and oral intubation methods. The RG method protocol used in the survival after WSSV challenge experiment did not elicit an infection level detectable by PCR and further improvement of the RG protocol is required to determine the potential for conducting disease challenges with a known virus titre delivered through a more natural infection route and for assessing immunostimulant products which show promise of viral adsorption with the digestive tract. Haemolymph samples taken for the immune response experiment were not analysed for WSSV concentration as per the survival experiment, therefore a low level WSSV infection deemed to be plausible due to decreases in THC and GHC.

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General Discussion

CHAPTER VI

6.1 Overview of thesis

This research investigated the application of fucoidans as a dietary intervention for common immune stressors in prawn farms. The experiments explored feeding commercially available fucoidan to the black tiger prawn (*Penaeus monodon*), to determine the immunostimulant effects of fucoidan on growth performance, feed efficiency, immune response, aerobic metabolism and survival. The black tiger prawn is the primary penaeid species cultured in Australia. The largest cause of production losses ($AUS10 M) is due to endemic viral outbreaks, namely Gill-Associated Virus (GAV) (Sellars et al., 2013). The majority of prawn production in Australia occurs in Queensland, where GAV is endemic to the region (Cowley et al., 2000; Munro et al., 2011). Limitations to expand the industry exist mainly due to the reliance on wild broodstock and the high costs associated with obtaining GAV-free stock from remote regions outside of Queensland (Preston et al., 2010; Mohr et al., 2015). Up to 100% of *P. monodon* are chronically infected with GAV under commercial conditions and transition to an acute infection due to environmental stressors such as changes in water temperature and salinity (Cowley et al., 2000; Spann et al., 2003; de la Vega et al., 2004; Elliot and Owens, 2015). Environmental stress reduces the immune vigour, such as decreased haemocyte counts, phagocytic activity, superoxide dismutase and modulation of phenoloxidase activity (PO activity) (Le Moullac and Haffner, 2000; Perazzolo et al., 2002).

Chapter 2 showed that fast growth rates and healthy digestive gland structures were supported when prawn feeds contained fucoidan at commercial inclusion levels; however there were no major changes in the immune response. Chapter 3 increased the fucoidan concentration of test ingredients in feeds and were fed to larger prawns for a longer duration. However there were no changes to the growth potential, although differences in total haemocyte count (THC) and granular haemocyte count (GHC) after prawns were subjected to simulated monsoon stressors of reduced temperature and salinity were measured. Therefore, in the following Chapter 4, a different approach was taken, by measuring the oxygen consumption of prawns during an environmental stress event, consisting of a combined decrease in water temperature and salinity. The aerobic metabolism of juvenile and sub-adult prawns decreased under simulated acute and chronic environmental stress; however juvenile prawns fed fucoidan had an increased AMR under acute environmental stress conditions. An increase in aerobic scope (A-SCOPE) may enable prawns to better cope with acute changes in environmental stress and provide an increased level of fitness for supporting oxygen-consuming functions such as fighting disease (Burnett et al., 2006; Thibodeaux et al., 2009). To further explore other applications of fucoidan as an ingredient in prawn feeds, Chapter 5 compared the effects of fucoidan and Sanictum® on the growth performance, immune response and survival post-infection with White Spot Syndrome Virus (WSSV), using IM and RG inoculation methods. Fast growth rates were supported by feeding fucoidan and Sanictum® to prawns, in comparison to decreased growth rates for prawns fed the Charoen Pokphand Foods (CPF) commercial feed treatment. Prawns inoculated by IM showed a decrease in immune response when infected with WSSV. However, the RG method did not manifest in an infection or overall change in immune response. When exposed to the severe challenge of WSSV, survival rates in IM inoculated prawns were low

indicating that feed treatments did not enhance survival, and high survival rates were seen in RG inoculated prawns, where WSSV load was below the level detectable by PCR.

6.2 Growth performance

Feeding immunostimulants have produced increased growth performance and feed efficiency in a range of prawn species (Smith et al., 2003; Cruz-Suarez et al., 2009; Traifalgar et al., 2010). It has been proposed that immunostimulants activate fixed phagocytes located within the hepatopancreas (DG), producing lytic enzymes which increase nutrient digestibility (Azad et al., 2005).The use of fucoidan products included in *P. monodon* feeds were investigated for their ability to increase growth performance and feed efficiency in Chapters 2 and 3, while selected fucoidan products and Sanictum® were compared in Chapter 5. In Chapter 2, purified fucoidan products (UP85, FV90 and F50) were included in juvenile *P. monodon* feeds at 0.2 $g.kg^{-1}$, according to commercial inclusion levels used by Chotigeat et al. (2004) to increase disease resistance in *P. monodon*, and MMB was included at 33 $g.kg^{-1}$ seaweed meal according to Cruz-Suarez et al. (2009). Although there were no differences in the growth performance and feed efficiency indices (WG, SGR, AWG, FI and FER) of prawns fed all feed treatments after 15 days of feeding; growth performance and feed efficiency was high, indicating there were no negative effects of fucoidan in feeds, and overall performance was equal to prawns fed the commercial reference feed (Ridley Aquafeed).

In Chapters 3 and 5, fucoidan inclusion levels were increased from 0.2 to 1 g. kg^{-1} according to recommendations within the literature (Traifalgar et al., 2010; Immanuel et al., 2012; Kitikiew et al., 2013) and Sanictum® was included only in Chapter 5 as a reference. In Chapter 2, the 0.2 g. kg^{-1} inclusion was adopted as a commercially viable option, while the increase in inclusion was adopted as a more effective experimental inclusion rate.

No differences were seen in either growth performance or feed efficiency between feed treatments. An increase in WG, SGR and protein retention was observed when *M. japonicus* were fed fucoidan at 0.5 and 1 g.kg^{-1} inclusion levels; while FCR was lower (Traifalgar et al., 2010). Our results indicate that fucoidan did not enhance growth performance or feed efficiency in three experiments, where fucoidan was fed at inclusion levels of 0.2 – 1 g.kg^{-1} for durations of 15-28 days. Furthermore, no changes in whole body crude protein and crude lipid were seen in the current experiment, this is in agreement with Traifalgar et al. (2010), who stated there were no changes in the whole body composition of *M. japonicus* after 8 weeks of feeding feeds containing fucoidan. An increase in lipid deposition would provide more efficient use of energy stores, and enhance protein accretion, sparing protein catabolism for metabolic energy production, as observed in red sea bream (*Pagrus major*) after feeding on seaweed meals (Yone et al., 1986; Nakagawa et al., 1997; Traifalgar et al., 2010).

6.3 Environmental stress

Fluctuations in extrinsic factors such as water temperature and salinity have a negative influence on the aerobic metabolism and immune response of prawns (Le Moullac and Haffner, 2000; de la

Vega et al., 2006). Prawn culture in Australia experiences high rainfall events which simultaneously decrease both water temperature and salinity; this stressor results in increased prawn mortality and significant reductions in productivity (de la Vega et al., 2006; Munro et al., 2011; Sellars et al., 2013). During commercial prawn production at the upper extreme, the water temperature can reach 30°C and salinity 40‰. However during periods of high rainfall such as the start of the monsoonal season, both water temperature and salinity can drop to 22°C and 10‰ within 2 to 3 days as a result of cooler rainwater mixing with the warmer seawater (Matt West, Pers. Comm.).

6.3.1 Aerobic metabolism

The effects of environmental stressors have been documented in prawn research (Chen and Nan, 1993; Le Moullac and Haffner, 2000; Perazzolo et al., 2002; Spanopoulos-Hernandez et al., 2005; Joseph and Philip, 2007). Metabolic depression is a reduction in aerobic metabolism during disease infection and is thought to be a due to the immune response impairing normal metabolic function (Burnett et al., 2006; Scholnick et al., 2006; Thibodeaux et al., 2009). At environmental extremes, the A-SCOPE of aquatic organisms has been shown to decline due to an inability of the cardiorespiratory system to supply oxygen to metabolically active tissues (Frederich and Portner, 2000; Portner, 2010; Sokolova et al., 2012). There is little known regarding the effect of dietary immunostimulants on aerobic capacity and corresponding ability to respond to stress events in prawns.

Soluble polysaccharide extracts from *Macrocystis pyrifera* have shown to maintain oxygen consumption rates in *Litopenaeus vannamei* infected with *Vibrio campbellii*, while control

prawns had a significantly lower oxygen consumption rate (Sanchez-Campos et al., 2010). Chapter 4 explored feeding immunostimulants prior to a simulated environmental stress and measuring the effect that chronic and acute environmental stress had upon the aerobic metabolism of juvenile and sub-adult *P. monodon*. The results determined that juvenile *P. monodon* fed 1 $g.kg^{-1}$ fucoidan had an increased active metabolic rate (AMR) when subjected to an acute environmental stress, when compared to the controls. Prawns fed the fucoidan feed treatment consumed an average of 0.59 mg g WW^{-1} h^{-1} of oxygen during active measurements, while prawns fed the basal feed consumed 0.50 mg g WW^{-1} h^{-1} oxygen. However, this effect was not profound enough to significantly influence A-SCOPE. In contrast sub-adult prawns subjected to the chronic environmental stress and fed the basal and FV90 treatments consumed on average 0.49 and 0.51 mg gWW^{-1} h^{-1} of oxygen respectively. In non-stress sub-adult prawns the AMR was higher than that in both juvenile and sub-adult stress groups, where average AMRs were 0.71 and 0.75 mg gWW^{-1} h^{-1} of oxygen for basal and FV90 fed prawns respectively; demonstrating that a combined decrease in water temperature and salinity had a negative impact upon prawns.

In both experiments, juvenile and sub-adult prawns tested positive for the presence of GAV, where viral load was up to 80 times greater in stressed sub-adult prawns fed the basal feed treatment, compared to the viral load of juvenile prawns fed both feed treatments. Previous research showed that adult *L. vannamei* infected with *V. campbellii* had a decreased oxygen consumption rate, which was half (24 mg $kgWW^{-1}$ oxygen) of the immunostimulant group (46 mg $kgWW^{-1}$ oxygen) and similar to pre-injection controls (Sanchez-Campos et al., 2010). The gills of crustaceans play important roles in the gas exchange, ion transport and immune defence

against pathogens (Smith and Ratcliffe, 1980; Burnett et al., 2006). In the case of Sanchez-Campos et al. (2010), the reduction in the oxygen consumption of *L. vannamei* was thought to be due to nodule formation within the gills, where haemocytes bind to the foreign particles, which affected oxygen uptake and subsequent hypoxia in unstimulated infected prawns. In contrast the prawns stimulated by either immersion or injection of soluble polysaccharides did not demonstrate a decrease in oxygen consumption (Sanchez-Campos et al., 2010). The immersion and injection stimulated prawns had increased total haemolymph proteins, and therefore had a higher concentration of antimicrobial peptides from haemocyanin to fight the infection. In the current study, there was no significant difference in the GAV load between juvenile prawns fed fucoidan or the basal feed, or between sub-adult prawns fed fucoidan or the basal feed. The main differences between these two experiments was the fact that juvenile prawns were subjected to an acute environmental stress (7 h), while sub-adult prawns were subjected to a chronic environmental stress (72 h). Acute changes in temperature effect biochemical reactions and A-SCOPE, limiting growth, reproduction and activity at extremes of high or low temperatures, as experienced in intertidal zones (Healy and Schulte, 2012). By acclimating animals to a chronic stress prior to measuring aerobic metabolism and A-SCOPE, it is possible to achieve a better understanding of the effects that temperature has on the aerobic metabolism (Fry and Hart, 1948; Healy and Schulte, 2012).

6.3.2 Immune response

Throughout this thesis, immune responses (THC, GHC and PO activity) have been measured consistently, under varying conditions. In Chapter 2, the THC, GHC and PO activity were

measured in prawns at the start and at the conclusion of the 15 day feeding experiment. Samples were pooled per tank to achieve enough haemolymph for the immune response analyses, due to the size of the prawns (average weight 4 g). The main findings of this experiment showed little differences in immune response of *P. monodon* fed all feeds. Prawns fed the FV90 had significantly higher GHC compared to prawns fed the F50, however this did not manifest to a higher PO activity. In fact, prawns fed the basal feed showed the highest PO activity (0.028 Δ Abs/min^{-1}), which was significantly higher than prawns fed the UP85, F50 and commercial reference feed (Ridley). It was proposed that the level of fucoidan may have been too low and the duration the prawns were fed these diets may not have captured peak immune response. These fucoidan ingredients minus the MMB were further assessed in Chapter 3.

In Chapter 3, prawns were fed fucoidan (FV90, UP85 and F50) at a higher inclusion rate (1 g.kg^{-1}), according to previous studies within the literature (Traifalgar et al., 2010; Immanuel et al., 2012; Kitikiew et al., 2013) for 21 days. At the conclusion of the feeding period prawns were subjected to an environmental stress for 3 hours (24°C and 20‰) or kept under optimal conditions (30°C and 40‰). Environmental stress causes significant reductions in haemocyte numbers (Le Moullac and Haffner, 2000; de la Vega et al., 2006; Wang and Chen, 2006; Pan et al., 2008) and PO activity (Perazzolo et al., 2002; Joseph and Philip, 2007; Traifalgar et al., 2010; Kitikiew et al., 2013), resulting in impaired immune function of prawns and or succumbing to disease infections caused by viral pathogens (Persson et al., 1987; Le Moullac and Haffner, 2000; Perazzolo et al., 2002; Liu et al., 2006; Joseph and Philip, 2007). In the current experiment, prawns subjected to acute environmental stress had significantly less THC (18.59%) and GHC (17.62%), compared to prawns kept under optimal conditions; demonstrating

the stress response caused a suppression of the immune system. Prawns fed fucoidan treatments did not differ in immune response, compared to prawns fed the basal feed treatment. PO activity was not different between feed treatments or stress/non-stress treatments. This was partly due to the high variation between individual animals, which were sampled over 6 days to ensure processing of individual samples were not compromised, however a significant sampling time effect was identified by three-way ANOVA.

The highest purity fucoidan (FV90) treatment was further explored in Chapter 4 in conjunction with an environmental stress, prior to measuring the aerobic metabolism in sub-adult prawns (average weight 19.08 g). *P. monodon* were fed for approximately 26 days to ensure prawns were in correct moult stage prior to chronic environment stress, where prawns were subjected to a combined decrease in water temperature and salinity from 30 to 24°C and 40 to 20‰, or kept at optimal water parameters (30°C and 40‰). Water temperature and salinity was decreased over a 7 h period before prawns were allocated a further 72 h to recover from the initial stress. The results of immune response when analysed by two-way ANOVA showed no significant differences. When data were pooled by stress/non-stress treatments, prawns subjected to the environmental stress showed increased THC (25%), GHC (39%) and PO activity (62%), compared to the non-stress treatments. In this Chapter, the immune response of prawns was determined after all measurements were taken for aerobic metabolism; including AMR where prawns were physically chased by hand until exhaustion. The immune responses in the current experiment are in fact the opposite of the results obtained in Chapter 3, where prawns subjected to environmental stress showed a decrease in THC and GHC. This may be attributed to measuring immune response post-chasing to exhaustion, where prawns held under optimal

parameters (30°C and 40‰) have an increased ability to utilise and regulate energy metabolism as a role for survival, where they in fact have a higher energy expenditure, accelerated by using more ATP for physical activity, such as escaping being chased by hand (Sokolova et al., 2012). Prior to sampling the immune responses, the non-stress sub-adult group were lethargic and showed gross signs of oxidative stress in the tail muscle, by the presence of opaque muscle tissue (Niu et al., 2013). Therefore, measuring the immune response after prawns have been subjected to a physical stress, such as chasing to exhaustion, may not accurately demonstrate the effect of the environmental stress, rather the effect of the chasing event.

In Chapter 5, *P. monodon* were fed feeds containing fucoidan (FV90 & UP85) and β-glucan (Sanictum®) for 28 d; prior to measuring the immune response (THC, GHC and PO activity) at the conclusion of the feeding experiment. There were no differences in THC, GHC or PO activity in prawns fed all feed treatments. However, it has been reported that immune responses (THC, PO activity and respiratory burst) increase with time when *L. vannamei* were fed feeds containing 1g.kg^{-1} (Kitikiew et al., 2013). This may be attributed to the experimental regime used in the current experiment, where a higher dose of fucoidan and Sanictum® may be more effective in provoking an immune response.

Five prawns from each feed treatment were then inoculated with WSSV by IM or RG methods, while control prawns were inoculated with a PBS buffer by IM or RG methods and served as controls. All prawns were sampled for THC, GHC and PO activity 24 h post-challenge. There were no differences in THC or GHC for prawns inoculated with WSSV by IM. When pooled by

inoculum type (WSSV or PBS), prawns infected with WSSV had 51% less THC and 33% less GHC compared to prawns inoculated with PBS. The PO activity of prawns inoculated with WSSV (IM) was not different between feed treatments. However prawns inoculated with WSSV and fed the basal and FV90 treatments had significantly lower PO activity when compared to prawns fed the basal and FV90 feeds in the PBS group. On the other hand, prawns fed UP85 and Sanictum had similar PO activity for both WSSV and PBS treatments, showing a limited or delayed response to the WSSV infection. Prawns fed UP85 and inoculated by the RG method showed a significant reduction in THC when compared to the PBS group. However, this decrease did not manifest in differences in GHC or PO activity.

In Chapter 5, haemolymph was only sampled for immune response at one time point (24 h post-challenge), therefore, further comparisons on the effect of time post-infection after 24 h cannot be determined. Haemolymph was not sampled at other points in the current research due to the increased numbers of prawns required for the analyses and facilities available.

6.4 Survival after infection with WSSV

First reported in 1992, WSSV has caused economic losses of more than $7US billion (Lightner, 2003). To date, WSSV is commonly used as a benchmark virus for assessing the efficacy of immunostimulant products in penaeid prawns (Chang et al., 1999; Chotigeat et al., 2004; Citarasu et al., 2006; Balasubramanian et al., 2008; Immanuel et al., 2012; Peraza-Gomez et al., 2014). In Chapter 5, a disease challenge was conducted where prawns were inoculated by IM and RG methods with a WSSV inoculum dose of 2.81 $x10^{8}$ copies/ml^{-1}, prepared following the

methods of Escobedo-Bonilla et al. (2005) and Rahman et al. (2006). The RG method was adopted to determine whether fucoidan may enhance survival of *P. monodon* by blocking viral adsorption of WSSV within the gut as proposed by De Somer et al. (1968) and Baba et al. (1988). Prawns inoculated with WSSV by IM had high mortality rates. Prawns fed the basal feed suffered 100% mortality by day 7, while prawns fed immunostimulants had 11.11% survival by day 7. On the contrary, *P. monodon* inoculated with WSSV by RG, observed prawns fed the basal treatment had 70% survival at day 21, while prawns fed FV90, UP85 and Sanictum had survival rates of 67 and 63% respectively; mortality in all prawns ceased by day 21. In a study by Immanuel et al. (2012), *P. monodon* were fed fucoidan at inclusion rates of 1, 2 and 3 $g.kg^{-1}$ for 45 days, prior to a WSSV challenge (IM); the survival percentage increased (50%) according to fucoidan inclusion. In the current study the low survival may be due to the pathogenicity of the WSSV stock used. The virus titre of diseased prawns was not different between prawns fed all feed treatments, inoculated by IM. For the RG group, the viral load of prawns inoculated by RG was either undetectable by RT-PCR or below the sensitivity of 20 copies/ml. Mortality is presumed to be due to low infection level or no infection, where virus may have been partially inactivated due to digestive enzymes and chemical barriers, before overwhelming susceptible epithelial cells (Escobedo-Bonilla et al., 2005). An increase in viral titre concentration by 1 $\log_{10}$ may reduce the severity of these digestive enzymes deactivating the WSSV inoculum (Escobedo-Bonilla et al., 2005). However, RG has been effective in inoculating prawns by pipetting a consistent dose of inoculum through the anus and hindgut to the midgut with positive infection and 100% mortality in *L. vannamei* with necrotizing hepatopancreatic bacterium (Aranguren et al., 2010).

6.5 Conclusion

There was limited advantage to including fucoidan in feeds for prawns at the sizes tested. Some responses and conditions under which stress and the severity of the challenge may have influenced the results obtained. Future work should consider including additional approaches such as molecular analyses, including heat shock protein analysis and immune-related gene expression of the prophenoloxidase system to measure the action of specific pathways at a molecular level during periods of environmental stress. The use of GAV-free stock in studies assessing aerobic metabolism will greatly expand on the results obtained in this study. Further improvement in the RG method is required to determine the potential of dietary fucoidan in viral adsorption in prawns.

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